EFFECT OF HYDROGEN SULPHIDE (H_2S) ON THE ELECTROPHYSIOLOGICAL PROPERTIES OF BOVINE ARTICULAR CHONDROCYTES

A Dissertation submitted in partial fulfillment of the requirement for the Degree of Doctor of Medicine in Physiology (Branch – V) Of The Tamil Nadu Dr. M.G.R Medical University, Chennai -600 032

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CERTIFICATE

This is to certify that the thesis entitled "**Effect of Hydrogen sulphide (H_2S) on the electrophysiological properties of bovine articular chondrocytes**" is a bonafide, original work carried out by Dr.N.Vasanthakumar, in partial fulfillment of the rules and regulations for the M.D – Branch V Physiology examination of the Tamil Nadu Dr. M.G.R. Medical University, Chennai to be held in May- 2018.

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DECLARATION

I hereby declare that the investigations that form the subject matter for the thesis entitled "**Effect of Hydrogen sulphide (H_2S) on the electrophysiological properties of bovine articular chondrocytes**" was carried out by me during my term as a post graduate student in the Department of Physiology, Christian Medical College, Vellore. This thesis has not been submitted in part or full to any other university.

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This thesis is dedicated to my guru – Otto Warburg.

Plagiarism certificate

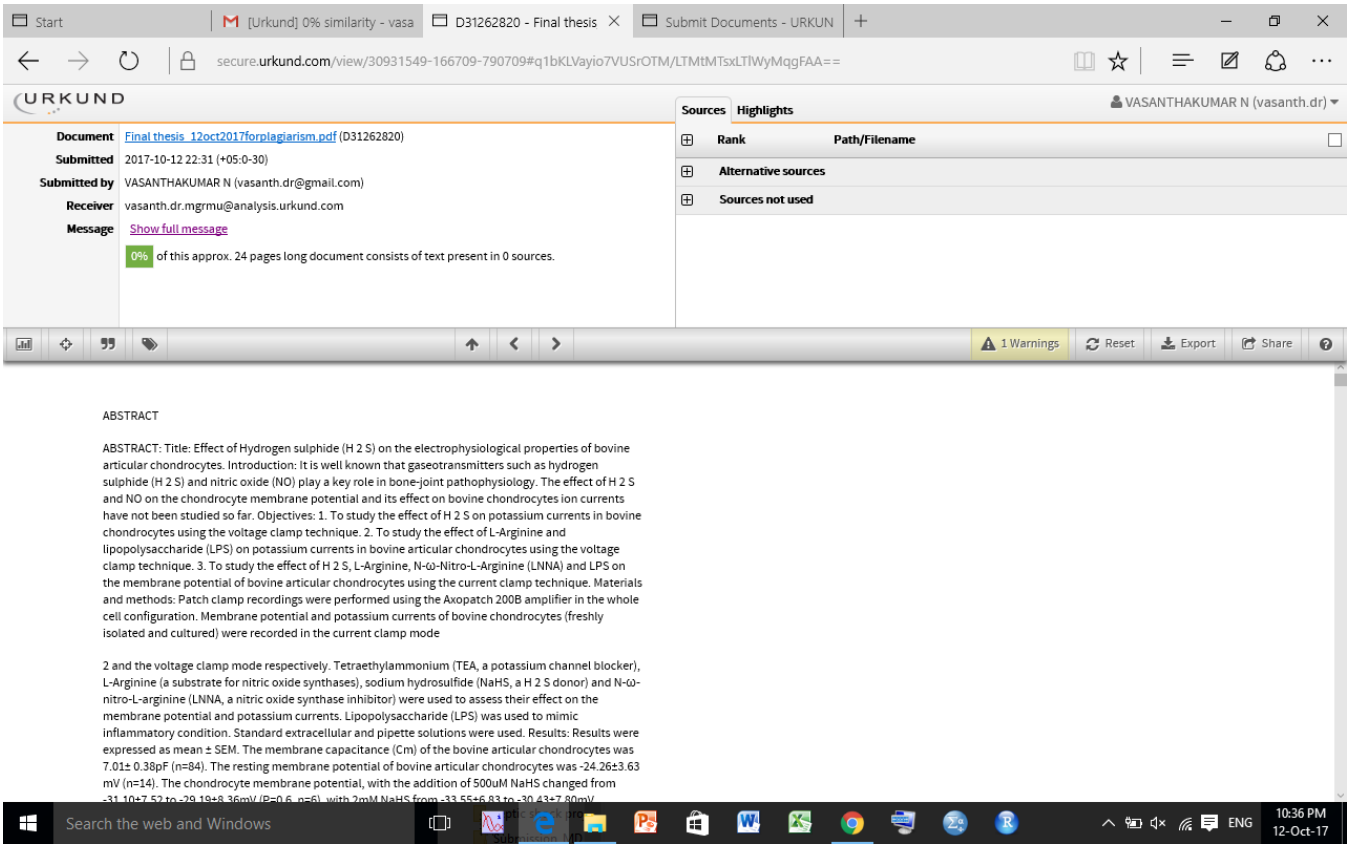


TABLE OF CONTENTS

ABSTRACT

ABSTRACT:

Title: Effect of Hydrogen sulphide (H_2S) on the electrophysiological properties of bovine articular chondrocytes.

Introduction: It is well known that gaseotransmitters such as hydrogen sulphide (H_2S) and nitric oxide (NO) play a key role in bone-joint pathophysiology. The effect of H_2S and NO on the chondrocyte membrane potential and its effect on bovine chondrocytes ion currents have not been studied so far.

Objectives:

1. To study the effect of H_2S on potassium currents in bovine chondrocytes using the voltage clamp technique.
2. To study the effect of L-Arginine and lipopolysaccharide (LPS) on potassium currents in bovine articular chondrocytes using the voltage clamp technique.
3. To study the effect of H_2S, L-Arginine, N-ω-Nitro-L-Arginine (LNNA) and LPS on the membrane potential of bovine articular chondrocytes using the current clamp technique.

Materials and methods:

Patch clamp recordings were performed using the Axopatch 200B amplifier in the whole cell configuration. Membrane potential and potassium currents of bovine chondrocytes (freshly isolated and cultured) were recorded in the current clamp mode

and the voltage clamp mode respectively. Tetraethylammonium (TEA, a potassium channel blocker), L-Arginine (a substrate for nitric oxide synthases), sodium hydrosulfide (NaHS, a H_2S donor) and N-ω-nitro-L-arginine (LNNA, a nitric oxide synthase inhibitor) were used to assess their effect on the membrane potential and potassium currents. Lipopolysaccharide (LPS) was used to mimic inflammatory condition. Standard extracellular and pipette solutions were used.

Results:

Results were expressed as mean ± SEM. The membrane capacitance (Cm) of the bovine articular chondrocytes was 7.01± 0.38pF (n=84). The resting membrane potential of bovine articular chondrocytes was -24.26±3.63 mV (n=14). The chondrocyte membrane potential, with the addition of 500uM NaHS changed from -31.10±7.52 to -29.19±8.36mV (P=0.6, n=6), with 2mM NaHS from -33.55±6.83 to -30.43±7.80mV (P=0.173, n=6), with 1 mM L-Arginine from -23.98±6.43 to -27.83±5.01mV (P= 0.374, n=9), with 10mM TEA from -24.99±3.67 to -20.21±3.26mV (P=0.221, n=14), with 2mM LNNA from -30.03±9.80 to 0.53±1.86mV (P=0.109, n=3).

Voltage clamp studies at 60 mV showed that the mean peak current density on addition of 500uM NaHS changed from 370.68±115.71 pA/pF to 411.93 ± 117.92 pA/pF (P= 0.3, n=14), with 2mM NaHS from 162.53 ± 100.83 pA/pF to 306.54 ± 148.42 pA/pF (P=0.144, n=4), with 8µg/ml LPS on the first exposure from 729.60 ± 248.20 pA/pF to 688.73 ± 239.05 pA/pF (P=0.465, n=4), with 8µg/ml LPS on the second exposure from 535.45 ± 218.69 pA/pF to 434.34 ±

103.34 pA/pF (P=0.593, n=3), with 10mM TEA from 249.53 ± 54.23 pA/pF to 139.93 ± 47.37 pA/pF (P=0.091, n=7). A tail current protocol showed a reversal potential (E_{rev}) of - 60mV.

Conclusion:

TEA results suggest that the currents seen in the bovine chondrocytes are due to the opening of voltage gated delayed rectifier potassium channels. A depolarization of membrane potential with TEA indicates that potassium channels may play a role in the maintenance of the chondrocyte membrane potential.

A depolarization of the membrane with LNNA suggests that Nitric oxide may also play a role in chondrocyte membrane potential regulation. Although the membrane potential and current density changes produced by NaHS, L-arginine and LPS were statistically insignificant, the trend observed suggests the need to further study this phenomenon with a larger sample size.

Keywords: Bovine chondrocytes, membrane potential, hydrogen sulphide, nitric oxide, LNNA, L-Arginine, Lipopolysaccharide.

INTRODUCTION

INTRODUCTION:

Chondrocytes are the only cells present in articular cartilage. The extracellular matrix synthesis by chondrocytes varies in response to various factors one of which is the mechanical load applied on the joint. The concentration of nutrients and oxygen is lower in the synovial fluid as compared to plasma, and as the synovial fluid is the source of oxygen and nutrients for the chondrocyte, the chondrocyte is therefore exposed to these low levels. Chondrocytes live in a hyperosmolar environment where the negative charge of the proteoglycans in the extracellular matrix predominantly contributes to this hyperosmolarity (1). Amin et al have shown that his hyperosmolarity offers a protective advantage to the chondrocytes in response to injuries (2).

The chondrocyte is traditionally considered to be dependent on anaerobic metabolism (1). However, this viewpoint is changing. Lee and Urban have shown the existence of a negative Pasteur effect (3) and Otte has demonstrated the presence of the Crabtree effect/Aerobic glycolysis in articular cartilage (4). Ali Mobasheri et al in their recent work have shown the importance of the Warburg effect in the normal chondrocyte metabolism and in osteoarthritis pathology (5).

The chondrocyte membrane potential is less negative as compared to other cells. This provides a protective advantage to the cell for cell volume regulation (6). Voltage gated delayed rectifier potassium channels, chloride channels, and transient receptor potential (TRP) channels have been shown to play a key role in the regulation of the articular chondrocyte membrane potential (6-9).

Gaseotransmitters such as H_2S and nitric oxide have been shown to play a key role in chondrocyte pathophysiology. H_2S has been detected in the synovial fluid of patients with rheumatoid arthritis (RA) and osteoarthritis (OA). The synovial fluid H_2S levels have been shown to be increased in patients with RA (10-13). When chondrocytes were exposed to lipopolysaccharides or IL-1β, this resulted in cell death which was prevented by H_2S (11).

Nitric oxide has been shown to play a key role in Rheumatoid arthritis (14) and osteoarthritis (15, 16). Nitric oxide has been shown to reduce extracellular matrix synthesis in arthritis and NOS inhibitors in this condition have shown beneficial effects (16,17).

The effect of H_2S, nitric oxide and LPS on the electrophysiological properties of bovine chondrocytes has not been studied so far. The aim of this study was to study the effect of these compounds on the chondrocyte membrane potential and the chondrocyte ionic currents using the patch clamp technique. LPS was used to mimic the inflammatory condition in the bovine chondrocytes used for these experiments.

This study helps in understanding the role of H_2S and nitric oxide in chondrocyte electrophysiology in both normal and inflammatory conditions.

REVIEW OF

LITERATURE

REVIEW OF LITERATURE:

Chondrocyte Physiology:

Chondrocytes are the only cells present in the articular cartilage. Chondrocytes make up only 5% of the volume of articular cartilage, the rest of the volume being comprised of extracellular matrix. Articular cartilage is avascular. The cartilage lacks lymphatic and nerve supply. Chondrocytes live in a harsh hypoxic and hyperosmolar environment and the exact mechanisms of how they survive in this environment are not fully known. The chondrocytes get a limited supply of nutrients and oxygen from the synovial fluid. Extracellular matrix synthesis is regulated by the articular chondrocytes in response to load patterns (1).

The chondrocyte metabolism is considered to be primarily due to anaerobic glycolysis (18). The extracellular matrix predominantly consists of water, proteoglycan and collagen fibers. Type II collagen is the predominant type present in articular cartilage. Among the proteoglycans, aggrecan is the predominant one. The negative charge of the proteoglycans attracts positively charged cations which in turn lead to a hyperosmolar environment. This also alters the ionic environment with increased cations and decreased anions. The extracellular environment of the cartilage is acidic and the level of acidity increases from the superficial zone of the cartilage to the deep zone. The attraction of hydrogen ions towards the negatively charged proteoglycans leads to increased hydrogen ion concentrations in the extracellular matrix. The

increased lactate production due to glycolysis also contributes to the acidity of the external environment (1).

The concentrations of extracellular ions present in the superficial and deep zone of articular cartilage (1) are presented in Table 1, along with the ionic composition of the ECF used in this study.

	Superficial Zone (mM)	**Deep Zone (mM)**	**ECF used in this study (mM)**
Na^+	240-270	300-350	140
K^+	7-9	9-12	5
Cl^-	60-90	50-100	147
Ca^{2+}	6-9	14-20	1
Mg^{2+}	(data not available)	(data not available)	1
Osmolarity	310-370	370-480	314 (calculated)
pH	7.1-7.3	6.9	7.4

Table 1: A comparison of the ionic concentration of the extracellular environment of the cartilage with the ECF used in this study.

Cartilage can be divided in to four zones which are: the superficial, the mid zone, the deep zone and the calcified zone. Cartilage is avascular and does not have lymphatic and nerve supply. Cartilage gets its oxygen, glucose and other nutrient supplies from the synovial fluid. Only the superficial zone is in contact with the synovial fluid.

1. **Superficial zone:**

In this zone, the chondrocytes are small, flat and the collagen fibers are orientated horizontally. The cell density is also high as compared to other zones. This zone makes up around 10-20% of the cartilage thickness.

2. **Middle zone:**

In this zone, the chondrocytes are less dense than the superficial zone and the collagen fibers are orientated obliquely. This zone makes up around 40-60% of the cartilage thickness.

3. **Deep zone:**

In this zone, the chondrocytes are less dense than the middle zone and the collagen fibers are orientated vertically. This zone makes up around 30-40% of the total cartilage thickness.

4. **Calcified Zone:**

This zone is in between the deep zone and the subchondral bone, and contains hypertrophic chondrocytes (18).

The effect of hyperosmolarity on chondrocytes:

Amin et al showed that hyperosmolarity plays a protective role in chondrocytes. They demonstrated that following cartilage injury, incubation in hyperosmolar saline as

compared to incubation in normal isotonic saline increased the survival of chondrocytes (2).

Chondrocyte resting membrane potential:

Normally the chondrocyte membrane potential is less negative as compared to other cells. This extra negativity may be a protective mechanism as the chondrocytes need to respond to extreme osmotic changes with minimal change in cell volume (6). The chondrocyte membrane potential has been shown to be regulated by potassium channels (7, 8), chloride channels (9) and TRPV5 channels (6).

Chondrocyte Metabolism:

As mentioned earlier, the metabolism of chondrocytes is traditionally considered to be due to anaerobic glycolytic metabolism. As the oxygen concentration is low in the synovial fluid (6-10%) (3), it was thought that this leads to anaerobic glycolysis by the Pasteur effect. A decreased oxygen concentration shifts the metabolism of the cells from oxidative phosphorylation to the glycolytic pathway, which is called Pasteur effect. Lee and Urban showed that in articular cartilage, a decreased oxygen concentration leads to decreased glycolysis (3), which implies that the little concentration of oxygen available in the environment of chondrocytes plays a crucial role in glycolytic metabolism. Otte showed the presence of the Crabtree effect/or aerobic glycolysis in articular cartilage (4). Ali Mobasheri et al in their recent review showed the importance of the Warburg effect in normal chondrocyte metabolism and in the pathology of osteoarthritis. They also demonstrated decreased mitochondrial

respiration and increased nitric oxide levels in relation to the Warburg effect in osteoarthritis (5).

Chondrocyte regeneration:

Cartilage is traditionally considered to lack regenerative potential. However, recent finding suggests a different picture. These findings have shown the presence of stem cells in the cartilage and these cartilage stem cells seem to play a crucial role in osteoarthritis, as reviewed by Jiang and Tuan (19). Pluripotent transcription factors such as OCT4 and Sox2 were shown to be present to a small extent in normal chondrocytes. These were also recently shown to be increased during the culture period and fracture healing (20).

Ion Channels in chondrocytes:

Chondrocytes have been shown to contain a large array of ion channels (21). The ion channels identified in chondrocytes so far include potassium channels: delayed rectifier potassium channels (K_v), K_{ATP} channels, calcium dependent big conductance (BK_{Ca}) and small conductance (SK_{Ca}) potassium channels and inward rectifier potassium channels, sodium channels: voltage gated sodium channels (VGSC) and epithelial sodium channels (ENaC). The presence of voltage gated calcium channels (VGCC) is not clear. Transient receptor potential channel (TRP) - a non-specific cation channel has been demonstrated on the chondrocyte membrane (21). The TRPV5 channel may play a role in maintaining the resting membrane potential of chondrocytes (6).

Potassium channels in chondrocytes:

The presence of delayed rectifier potassium channels (K_v) in the articular chondrocytes has been shown by many researchers and has been reviewed by Ali Mobasheri et al (34). Delayed rectifier potassium channels were shown to be inhibited by TEA and these channels may play a key role in chondrocyte membrane potential regulation. The presence of K_v channels has been shown in goat articular cartilage in our lab (22).

Inward rectifier potassium channels

Ali Mobasheri et al showed the presence of K_{ATP} channels in chondrocytes. K_{ATP} channels belong to the inward rectifier potassium channel family and can be inhibited by ATP and glibenclamide (23).

Big K_{ca} and small K_{ca}

Big K_{ca} has been shown in articular chondrocytes and can be inhibited by specific blockers such as iberiotoxin. Small K_{ca} has also been shown in articular chondrocytes and can be inhibited by specific blockers such as apamin.

Leak potassium channels:

Leak K^+ channels have also been demonstrated in articular chondrocytes and they play a key role in maintaining the resting membrane potential (24).

Voltage gated sodium channels

Only a few studies have shown the presence of voltage gated sodium channels in articular chondrocytes. Sugimoto et al demonstrated the presence of these channels in articular chondrocytes and identified it as sensitive to the voltage gated sodium channel blocker Tetrodotoxin (TTX) (25).

Voltage gated proton channels:

Presence of voltage gated proton channels has been shown in the articular chondrocytes and they were inhibited by Zinc (26).

Chloride channels in articular chondrocytes:

Chloride channels have also been demonstrated on the chondrocyte membrane, and the presence of these channels has been suggested to play a key role in the regulation of chondrocyte membrane potential (9, 25).

The Patch clamp technique:

The patch clamp technique permits the user to perform both voltage clamp and current clamp studies. Voltage clamping holds the membrane potential of a cell or a small patch of the cell membrane at a desired voltage, where the ionic currents that activates at that specific voltage can be studied. Current clamping clamps the current at a desired level and the membrane potential effects of the cell can be recorded at the clamped level. When the current clamp is maintained at zero there will be no net ionic currents and one can record the resting membrane potential (RMP) of the cell.

A gentle suction applied after the patch pipette makes contact will result in giga seal and the membrane resistance would be in the order of giga ohms. Many patch clamp configurations exist (27), some of them are listed below,

1. **Cell attached patch clamp configuration:**

Once the giga seal is formed, the whole cell is tightly attached to the patch pipette tip. Using this configuration, one can study the ionic currents at the patch area only. The pipette solution should contain the ECF.

2. **Whole cell patch clamp configuration:**

After obtaining the giga seal, further application of mild suction will break the patch area without compromising the giga seal. In the whole cell mode we have access to the cell interior of the cell. The pipette solution usually should be similar to the intracellular fluid composition and the bath solution should be similar to the ECF. The whole cell patch clamp configuration is used to record the total population of ionic currents.

The whole cell patch clamp configuration was used for this study. The electrical circuit of this configuration is given in Figure 1.

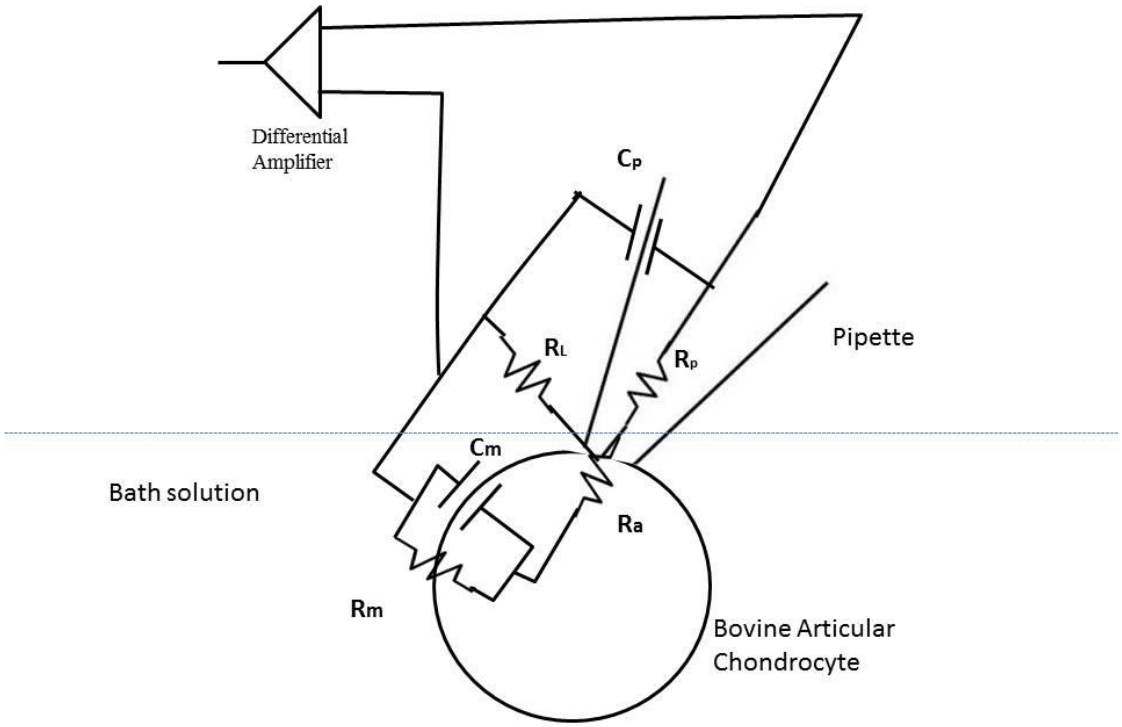


Figure 1: The electrical circuit of the whole cell patch clamp configuration: Membrane capacitance (C_m), pipette capacitance (C_p), seal (leak) resistance (R_L), pipette resistance (R_p), access resistance (R_a), membrane resistance (R_m).

3. Inside out patch clamp configuration:

After obtaining the cell attached configuration, a gentle pull will result in the formation of small vesicle at the patch pipette tip. When the pipette is pulled further from the bath and kept in the air for a short period, the vesicle will rupture and form the inside out patch clamp configuration. The pipette solution usually should be similar to the ECF and the bath solution should be similar to the intracellular fluid composition. The inside out patch clamp configuration is used to record single ion channel currents.

4. **Outside out patch clamp configuration:**

After obtaining whole cell configuration, a gentle pull will result in the formation of outside-out patch clamp configuration. Pipette solution should be similar to the intracellular fluid composition and the bath solution should be similar to the ECF. Outside-out patch clamp configuration is used to record single ion channel currents.

5. **Perforated patch clamp configuration:**

This configuration is similar to the cell attached configuration except that the pipette solution contains a perforating agent such as nystatin, which makes perforations in the patch membrane.

Hydrogen sulphide (H_2S):

H_2S is one of three key gaseotransmitters present in the body, the other two being nitric oxide (NO) and carbon monoxide (CO). It is synthesized in the body by the following three enzymes using L-cysteine, L-cystathionine and L-homocysteine as substrates:

- cystathionine β-synthase (CSE),
- cystathionine γ lyase (CSE) and
- 3-mercaptopyruvate sulfurtransferase (MST).

The physiological levels of H_2S in circulation are 10 – 100μM. An altered H_2S level has been shown to be associated with a large variety of pathological conditions (28).

H_2S and inflammatory joint disease:

H_2S has been detected in the synovial fluid of patients suffering from rheumatoid arthritis (RA) and osteoarthritis (OA) (10, 11). In RA patients, the synovial fluid H_2S levels has been shown to be increased (12, 13).

Although the role of nitric oxide as a proinflammatory mediator in joint pathologies is well known (16), the role of H_2S in joint pathologies is not very clear, as some studies have shown it to act as a proinflammatory mediator (13,29) while others have shown H_2S to act as an anti-inflammatory mediator (11,30). It has been shown that chondrocytes when exposed to lipopolysaccharide (LPS), it resulted in increased expression of CSE which resulted in H_2S synthesis and this H_2S prevented chondrocyte cell death (11).

H_2S and ion channels:

H_2S has been shown to interact with a wide variety of ion channels– K_{ATP} channels, BK_{Ca} -big conductance calcium activated potassium channel channels, SK_{Ca}- small conductance calcium activated potassium channel channels, Cystic fibrosis transmembrane conductance regulator (CFTR) chloride channels, voltage gated calcium channels, the transient receptor potential channel etc (28). The presence of KATP channels in chondrocytes has been shown by Mobasheri et al (23).

Nitric Oxide in Joint inflammation:

Nitric oxide is synthesized by nitric oxide synthases (NOS) from the substrate L-Arginine. Three types of nitric oxide synthases are present:

1. endothelial nitric oxide synthases (eNOS or NOS1),
2. inducible nitric oxide synthases (iNOS or NOS2),
3. neuronal nitric oxide synthases (nNOS or NOS3),

While eNOS and nNOS are constitutive enzymes, iNOS is an inducible enzyme present in conditions such as inflammation. Nitric oxide has been shown to play a key role in Rheumatoid arthritis (14). Nitric oxide has been shown to be involved in arthritis and a reduced extracellular matrix synthesis. The use of NOS inhibitors has shown beneficial effects in this condition (16, 17). Nitric oxide levels have been shown to be increased as a result of iNOS activation in osteoarthritis (15, 16). It is well known that NO is increased in sepsis (31).

Lipopolysaccharides (LPS):

Lipopolysaccharides (LPS) are components of the Gram negative bacterial outer cell membrane and are used extensively to mimic sepsis. It is well known that LPS increases the gaseotransmitters: nitric oxide and H_2S (33). It is also known that LPS acts as a pathogen associated molecular pattern which acts through the Toll-like receptor 4 and activates Nuclear Factor Kappa beta (NFkB) and iNOS which leads to increased nitric oxide levels.

While the role of nitric oxide as a proinflammatory mediator in sepsis is well known (31), the role of H_2S is not clear. H2S has been shown to produce anti-inflammatory effects in the cytokine stimulated chondrocytes by decreasing nitric oxide levels and decreasing inducible NOS expression (30). H_2S has been shown to decrease proinflammatory cytokines in LPS sepsis models (32). Some studies on the other hand

have shown the proinflammatory role of H_2S in Rheumatoid arthritis (13). H_2S plasma concentrations have been shown to be increased in patients with septic shock. H_2S has been shown to play a proinflammatory role in in-vivo sepsis animal models (33).

Current literature, to the best of our knowledge does not clearly explain the role of gaseotransmitters in chondrocyte electrophysiology. The understanding of these specific effects could add to our understanding on the role of these gaseotransmitters in normal chondrocyte physiology as well as various joint pathologies.

This study aims to identify the role of the gaseotransmitters, NO and H_2S, specifically on the electrophysiological properties of chondrocytes.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES:

Aim: To study the effect of the Hydrogen sulphide (H_2S) on the electrophysiological properties of bovine articular chondrocytes.

Objectives:

1. To study the effect of H_2S on potassium currents in bovine articular chondrocytes using the voltage clamp technique.
2. To study the effect of L-Arginine and Lipopolysaccharides (LPS) on potassium currents in bovine articular chondrocytes using the voltage clamp technique.
3. To study the effect of H_2S, L-Arginine, N-ω-Nitro-L-Arginine (LNNA) and LPS on the membrane potential of bovine articular chondrocytes using the current clamp technique.

MATERIALS AND METHODS

Materials and Methods

1. Chemicals used

Sodium hydrosulfide hydrate (NaHS) was used as a H_2S donor. L-Arginine was used as a substrate for nitric oxide synthases. N-ω-Nitro-L-Arginine was used as an inhibitor of nitric oxide synthases. Lipopolysaccharides (LPS – Escherichia coli strain O127:B8) was used to mimic inflammatory conditions. Dulbecco's modified Eagle's Medium(DMEM)/Nutrient mixture F-12 Ham with L-Glutamine and 15mM HEPES was used as the culture medium with added ascorbic acid 62 mg/L, antibiotics – penicillin, streptomycin (Penstrep 250 µg/ml – 10 ml/L) and amphotericin B (250 µg/ml – 8 ml/L) in the bovine articular chondrocyte preparation. Worthington Collegenase Type II was used for digesting the Bovine cartilage shavings. Tetraethylammonium (TEA) was used as a potassium channel blocker. All chemicals except Collagenase II were purchased from Sigma Aldrich.

2. Isolation of Bovine Chondrocytes

a) Harvesting bovine cartilage:

Skinned and de-hooved bovine legs were obtained from the local slaughter house within 4 hours of the death of the animal. A total of six different bovine animal legs were used in this experiment. Under aseptic conditions the metatarsophalangeal joints of the bovine legs were opened. Cartilage was shaved from the articular surface using a sterile surgical blade.

As mentioned earlier, Dulbecco's modified Eagle's medium (DMEM)/ Nutrient mixture F12 (1:1) was used as the culture medium, with added ascorbic acid 62 mg/L, antibiotics – penicillin, streptomycin (Penstrep 250 µg/ml – 10 ml/L) and amphotericin B (250 µg/ml – 8 ml/L).

Cartilage shavings were collected in a 15ml centrifuge tube with DMEM medium and washed twice in DMEM. The shavings were then kept in a culture flask containing 10 ml of DMEM for digestion. Cartilage not being digested was stored in 10ml of DMEM in a culture flask until the digestion step. The culture flask medium was changed every 2 days until the digestion step. Generally, the cartilage shavings were used within a week, except for one batch where the cartilage shavings were kept for one month.

b) Digestion of cartilage shavings:

Worthington Collagenase type II (335 Units/mg) was used in 2 mg/ml concentration for digesting the cartilage. 20 mg of the enzyme (equal to 6700 units) was mixed with 10ml of DMEM medium. This was added to the culture flask after passing through a 0.22µ syringe filter. Washed cartilage shavings were added in this flask and kept in a CO_2 incubator overnight for about 15 to 20 hours for digestion.

Digestion was arrested by adding double the amount of DMEM to the culture flask. The cells were strained using a 40 µM cell strainer and centrifuged at 2400 rpm for 10 minutes. The supernatant was discarded and the pellets were washed with DMEM. Finally, 5ml of DMEM was added to the pellet and gently triturated to make a cell suspension. The viability of the cells was confirmed by performing a trypan blue dye

exclusion test. Cells from this suspension were used for Patch clamp experiments and were also cultured as a monolayer in the culture flask for one day.

c) Monolayer cultures:

Cells were plated in a T25 culture flask and kept in a CO_2 incubator until harvesting. The culture medium was changed every 2 days. The primary monolayer culture was generally used after trypsinization within a week.

d) Harvesting cultured chondrocytes:

After one day, the cultured cells were harvested using 0.25% Trypsin with EDTA for 5 min. The digestion was arrested by adding double the amount of DMEM. This was collected in a 50 ml tube and centrifuged at 2400 rpm for 10 minutes. The supernatant was discarded and the pellet as washed twice with DMEM. 5ml of DMEM was added to the pellet and gently triturated to obtain the cell suspension for patch clamp studies. Cell viability was checked with trypan blue dye exclusion test.

3. Extracellular and Pipette solutions

Both the extracellular solution and the pipette solution were prepared using de-ionized water (Milli-Q). The extracellular solution was used as the patch clamp bath solution. The pipette solution was made with values close to those of the intracellular ionic environment. The contents of the extracellular solution and the pipette solution solutions are listed in Table 2 and 3 respectively.

Table 2: Extracellular solution composition

Compound	Concentration in mM
NaCl	140
KCl	5
$CaCl_2$	1
$MgCl_2$	1
HEPES	10
Glucose	10
pH adjusted to 7.4 with NaOH	

Table 3: Pipette solution composition

Compound	Concentration in mM
KCl	140
$MgCl_2$	1
HEPES	10
Glucose	10
pH adjusted to 7.3 with KOH	

The required concentrations of the salts were added to the de-ionized water and were mixed using a magnetic stirrer. The pH of the extracellular solution was adjusted to 7.4 using 1M NaOH and similarly the pH of the pipette solution was adjusted to 7.3 using 1M KOH.

4. Patch pipette preparation:

Pipettes used for the patch clamp recordings were fabricated using borosilicate glass tubes (Dimension: 1.5 – 1.8 X 100mm) from Kimble Chase. The ends of the borosilicate glass tubes were fire polished slightly to make it fit snuggly in the pipette holder. The fire polished borosilicate glass tube was kept in the pipette puller in such a way that its middle portion was surrounded by the heating filament. A Narshige (Japan) Vertical pipette puller was used to pull the pipettes, shown in Figure 2. It is a two-step gravity assisted vertical puller. The final result is two pipettes with fine tips from each glass tube. The pipettes made had a resistance of near 2MΩ. This was tested during patch clamp recordings after the pipettes were filled with pipette solution and they entered the bath solution.

Figure 2: Narshige Vertical Pipette puller.

The fine tips of the pipettes were fire polished using a GlasswoRX F-500 microforge. Fire polishing makes the pipette tips smoother and helps in getting good seals with the cells.

The area close to the pipette tips but excluding the pipette tips were coated with silicone elastomer (Sylgard, Dow Corning). Sylgarding reduces the pipette capacitance and also reduces noise.

Around 20 pipettes were prepared on the day of recording or one day before the recording. The pipette tips were backfilled with the pipette solution and later filled with the pipette solution to 2/3rd of the tube using a custom made plastic syringe. The pipettes were gently tapped to remove the trapped air bubbles in the pipette. The pipettes were then placed finally in the pipette holder which contained a chloride coated silver electrode and tightened.

4. Patch clamp setup:

a) Faraday Cage: All the patch clamp recordings were performed inside the Faraday cage to avoid electromagnetic noise interference. The Faraday cage used is shown in Figure 3.

b) Inverted microscope: Nikon Eclipse TE2000-U inverted microscope was used for visualizing the bovine chondrocytes kept in the center of a 35 mm petri dish filled with 2ml of the ECF as bath solution. Under 10X magnification the perfusion system pipette tip was kept close to the chosen chondrocyte which was positioned in the center of the visual field. Under 40X magnification, the patch pipette filled with pipette solution was moved to make contact with the chondrocytes.

c) Octaflow Perfusion system: An eight channel perfusion pump system controlled by the Octaflow II Digital valve control system (ALA Scientific Instruments) was used to deliver the necessary solutions to the recording bovine chondrocytes kept in

the 35mm petri dish on the inverted microscope platform. Nitrogen from a nitrogen cylinder was used as a pressure source.

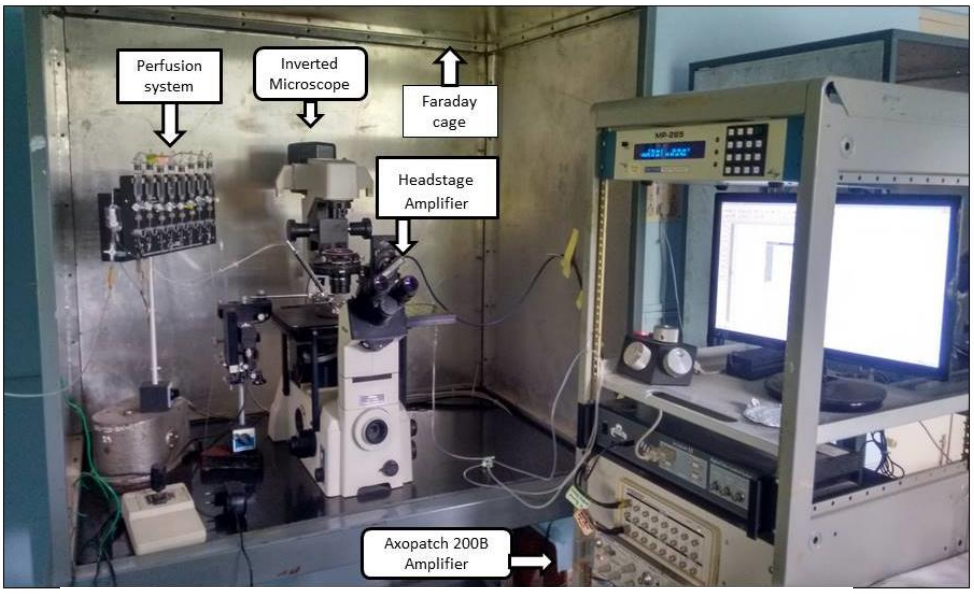


Figure 3: Patch Clamp recording setup

d) Micromanipulator: The solution filled pipette was kept in the pipette holder which had a chloride coated silver electrode. The pipette holder was fixed with the CV 203BU head-stage (Axon Instruments), which in turn was connected to the Axopatch 200B amplifier through a 16 bit resolution Analog to Digital convertor – Digidata 1322A (Axon Instruments) which had a maximum sampling rate of 500KHz. The pipette was manipulated using a MP–285 Micromanipulator (Sutter Instruments) to position the pipette to touch the focused bovine chondrocyte. After gently touching the chondrocyte (Figure 7), a gentle suction was applied to make a giga seal (GΩ resistance seal) through a small tube attached to the pipette holder.

e) Axopatch 200B amplifier: An Axopatch 200B amplifier was used for the patch clamp recordings. A low pass filter of 5 KHz was used for the voltage clamp recordings and a low pass filter of 2 KHz was used for current clamp recordings. Offset, pipette capacitance transient cancellation, series resistance and membrane resistance cancellation were done through the respective functional knobs present in the Axopatch 200B amplifier. A Gain of 1 was generally used except for the chondrocytes which had high outward currents where the gain was reduced to 0.5. The pClamp 9.2 software– which contained Clampex for data acquisition and Clampfit for data analysis was used.

g) Seal test: The seal test function in Clampex was used to calculate the pipette resistance after the pipette entered the bath solution. This was close to 2 MΩ generally. After the giga seal was made with the focused bovine chondrocyte, the seal was broken by gentle suction. The membrane test function of Clampex was used to automatically calculate the following parameters:

- membrane capacitance (Cm),
- seal resistance (Rseal),
- access resistance (Ra),
- time constant (Ƭm) and
- holding current.

f) Pulse Protocol: The stimulus waveforms for the voltage clamp and current clamp recordings were made using Clampex. A representative pulse protocol used in the voltage clamp recordings is shown in Figure 4.

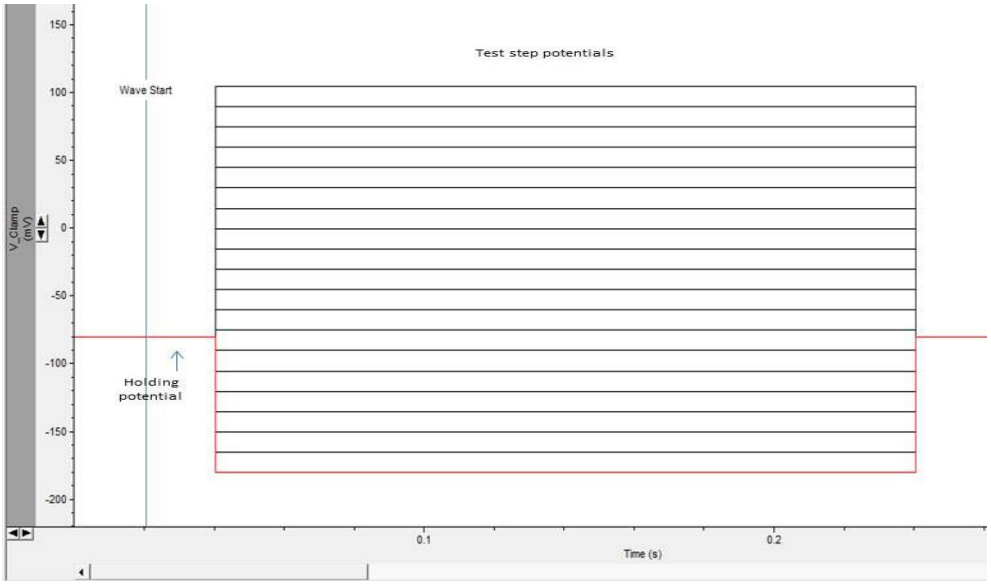


Figure 4: One of the pulse protocols used in voltage clamp recordings. Holding potential was -80mV and the test step pulses range from -180mV to 105mV in 15mV steps. Each test step pulse duration was 200 milli seconds.

Reversal potential (E_{rev}) calculation using tail current pulse protocol:

The reversal potential **(E_{rev})** was calculated using the tail current pulse protocol shown in Figure 5.

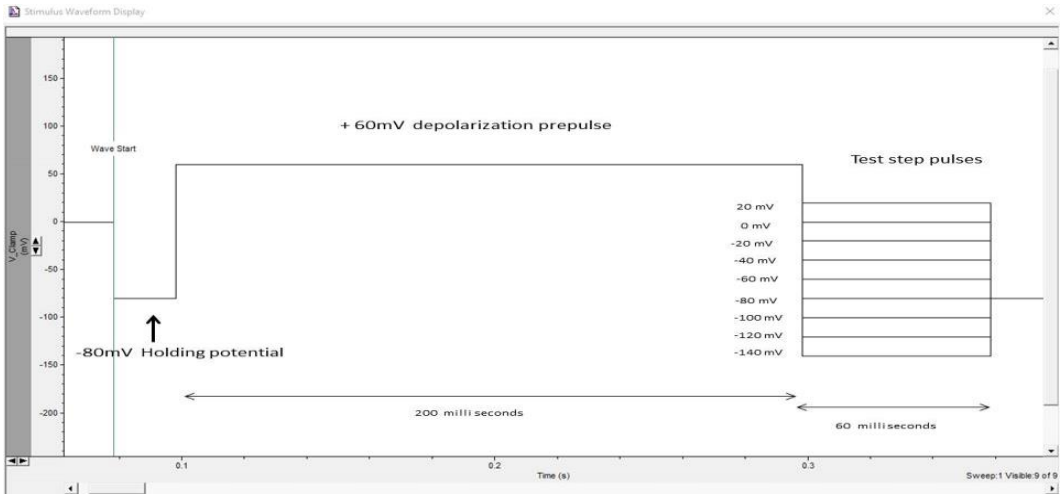


Figure 5: Tail current pulse protocol

From the holding potential of -80mV, a depolarization step at +60mV was applied for 200 milli seconds. The depolarization pre-pulse step will generally activate all types of ion channel currents in the chondrocytes. At the end of depolarization pre-pulse, a series of test step pulses were applied from -140mV to +20mV in 20mV steps for 60 milliseconds, which makes up a total of nine sweeps. The reversal potential of the bovine chondrocytes was expected to be in the test pulse range. Stepping down from the depolarization pre-pulse step to test step pulses will reduce the currents and the direction of the current flow in the time period just after the depolarization step will give the indication of the reversal potential. A time segment of about 5 milliseconds was chosen just after the test pulse was started. The current flow at each voltage is recorded and the voltage where the current flow was zero was taken as the reversal potential.

The Nernst equilibrium potential for potassium ions:

We also calculated the Nernst equilibrium potential for potassium ions from the values of the potassium ion concentrations in the extracellular fluid and pipette solution using Nernst equation.

$$E_k = \frac{RT}{ZF} \; ln \; \frac{[K^+]_0}{[K^+]_i}$$

E_k - Nernst Equilibrium potential for potassium ion

R - Universal gas constant

T - Temperature in kelvin (Room temperature was 24^0C and T = 297.15 K)

Z - valence of the ion

ln - natural logarithm

$[K^+]_O$ - Potassium ion concentration in extracellular fluid

$[K^+]i$ - Potassium ion concentration in intracellular fluid/pipette solution

The calculated equilibrium potential for potassium ion (E_k) using the Nernst equation for equilibrium potential was found to be equal to – 85.32mV for the solutions used.

Current clamp pulse protocol:

For the current clamp recordings, the low pass filter was set to 2KHz in the Axopatch 200B amplifier. The current clamp protocol used is shown in Figure 6.

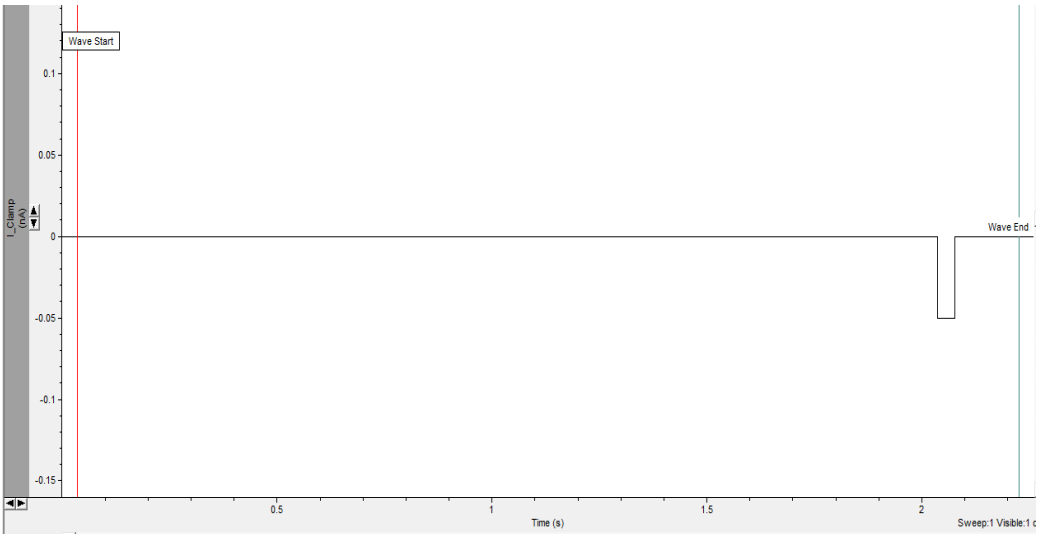


Figure 6: Current clamp protocol: Each sweep duration was 2.3 seconds.

Each Current clamp sweep duration was 2.3 seconds. The current clamp protocol maintained zero current except for a small region near the end of the sweep at 2 seconds, where a -0.05nA current injection pulse step was applied for 40 milliseconds

to check the seal stability. For each test condition about10 sweeps were chosen and the average of the traces was taken. From the averaged trace, a 100 millisecond time segment was chosen just before the current injection pulse step and the mean membrane potential for each test condition was calculated.

Electrophysiological recording of Bovine chondrocytes:

From the bovine chondrocyte cell suspension (made either freshly or from the primary monolayer culture) ~ 100uL of the cell suspension was placed in the center of a 35mm petri-dish and smeared gently in the central area. It was kept in the incubator for 15 minutes to allow the chondrocytes to adhere. After 15 minutes the petri-dish was filled with 1ml DMEM in the laminar hood and placed again in the incubator till its use in patch clamp recordings. Generally these petri-dishes were used in one or two days.

The petri-dishes were then used for recording. The culture medium was first removed and the cells were washed gently with ECF. The petri-dish was then filled with 2ml of ECF which acted as the bath solution. The petri-dishes were placed on the inverted microscope platform. A silver/silver chloride electrode which acted as a reference electrode was placed in the bath solution. Under 40X magnification the bovine chondrocytes were visualized and after choosing a healthy looking bovine chondrocyte, it was positioned in the center of the field (Figure 7). The perfusion system pipette was placed close to the focused cell under 10X magnification. The pipettes filled with pipette solution were placed in the pipette holder and tightened.

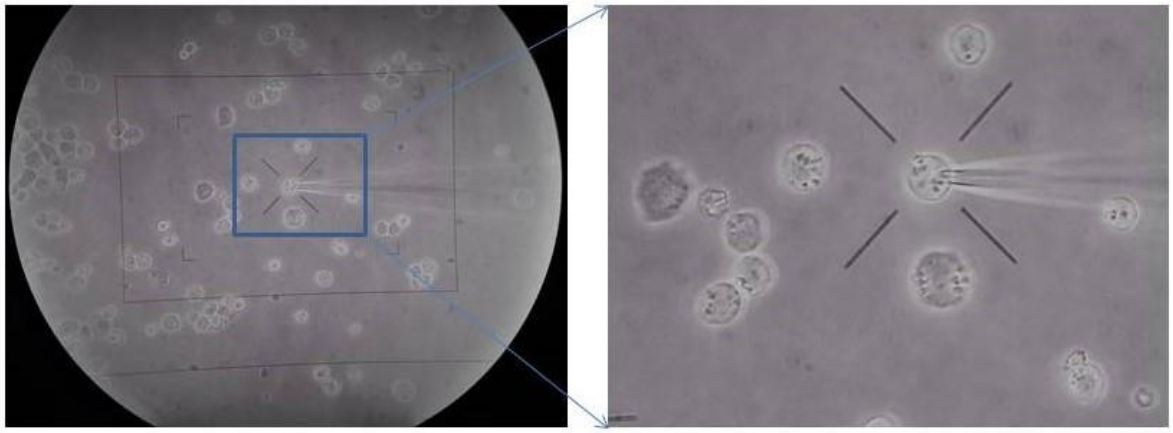


Figure 7: Bovine chondrocytes visualized under 40X. The center of the field was expanded and shown in the right side box. A gigaohm (GΩ) sealed Bovine chondrocyte with the pipette tip can be seen.

The pipette position was adjusted using the micromanipulator. Once the pipette tip touched the bath solution, the pipette resistance (R_p) was obtained using the seal test function. This was usually found to be around 2MΩ. The offset was corrected using the offset correction knob in the amplifier. The pipette was further lowered down to touch the focused chondrocyte and a giga seal was made by gentle suction. Pipette capacitance transients were cancelled using the pipette capacitance transient correction knob in the amplifier.

After the giga seal was obtained, the seal was ruptured by a further gentle suction. Using the Membrane test function, the capacitance of the bovine chondrocyte membrane (Cm in picofarads) was obtained.

For voltage clamp studies, the low pass filter was set to 5KHz. Usually the amplifier gain was set to one, but if the currents were large and saturating the amplifier gain was reduced to 0.5. The membrane capacitance transients and the series resistance was

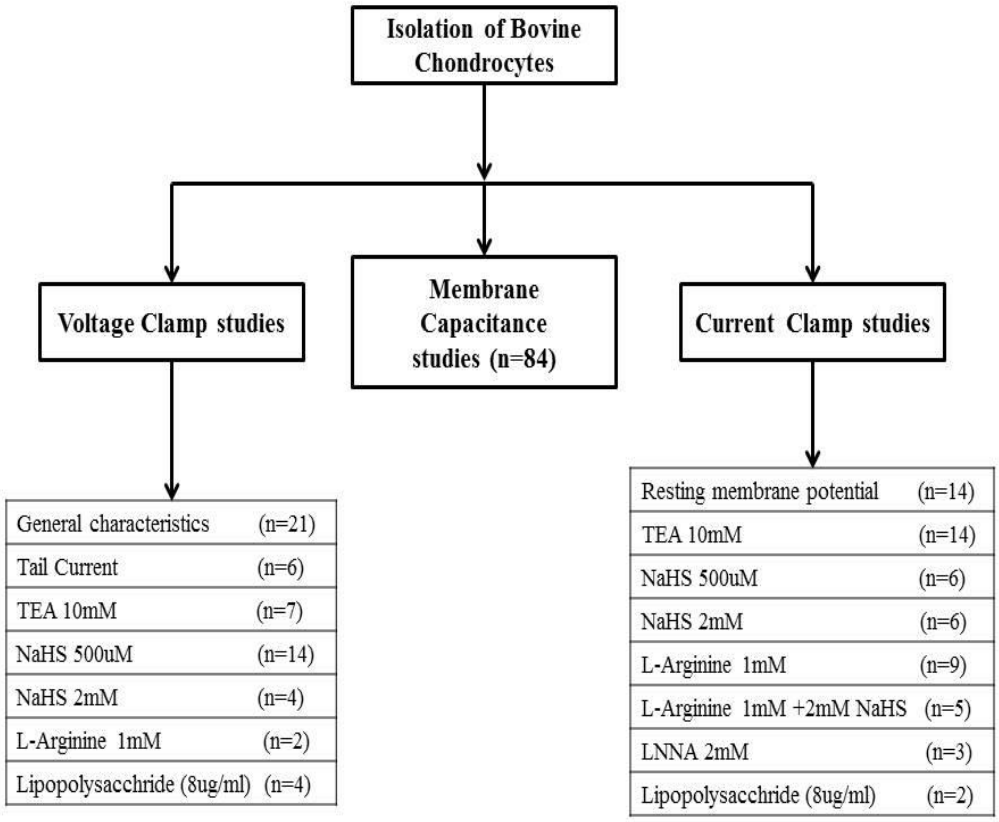
compensated (40 to 60%) by the series capacitance and membrane capacitance cancellation knobs in the amplifier. The currents were then recorded using the required voltage clamp pulse protocol under the required perfusion condition.

For current clamp recordings, the low pass filter was set to 2 KHz and the amplifier was switched to the current clamp mode after the membrane test function. The chondrocyte membrane potential was then recorded using the current clamp pulse protocol under the required perfusion conditions.

5. Statistical Analysis.

Electrophysiological data were transferred from Clampfit to Microsoft Excel 2010. The values of each test are presented as Mean ± SEM using Excel. Figures were produced using IgorPro 5.0.4.8 (Wavemetrics Inc). Statistical significance was tested using the Wilcoxon Signed Ranks test. A P value less than 0.05 was considered significant. Statistical significance was tested using IBM SPSS Statistics 21.0.0.

6. Experimental Design Flow chart



RESULTS

RESULTS:

Both freshly isolated bovine chondrocytes and cultured chondrocytes after trypsinization from primary monolayer culture were used in the patch clamp experiments. These bovine chondrocytes were obtained from six different animals. The results are presented under the following section headings.

I**. Membrane Capacitance results.**

II. **Voltage Clamp experiments results.**

A. General characteristics of the bovine chondrocytes.
B. Tail Current results.
C. TEA results.
D. NaHS results.
E. L-Arginine results.
F. LPS results.

III. Current clamp experiments results.

A. Resting membrane potential of the bovine chondrocytes.
B. TEA results.
C. NaHS results.
D. L-Arginine results.
E. NaHS + L-Arginine results.
F. LNNA results.
G. LPS results.

I. Membrane Capacitance results:

Membrane capacitance (Cm) was calculated using the membrane test function in Clampex immediately after the giga ohm seal was broken. Cm was measured in pico farad units (pF). A representative membrane capacitance tracing is shown in Figure 8. Mean and SEM (standard error of Mean) of the membrane capacitance (Cm) was calculated from 84 bovine chondrocytes (freshly isolated and cultured). The mean Cm recorded was 7.01±0.38 pF (n=84).

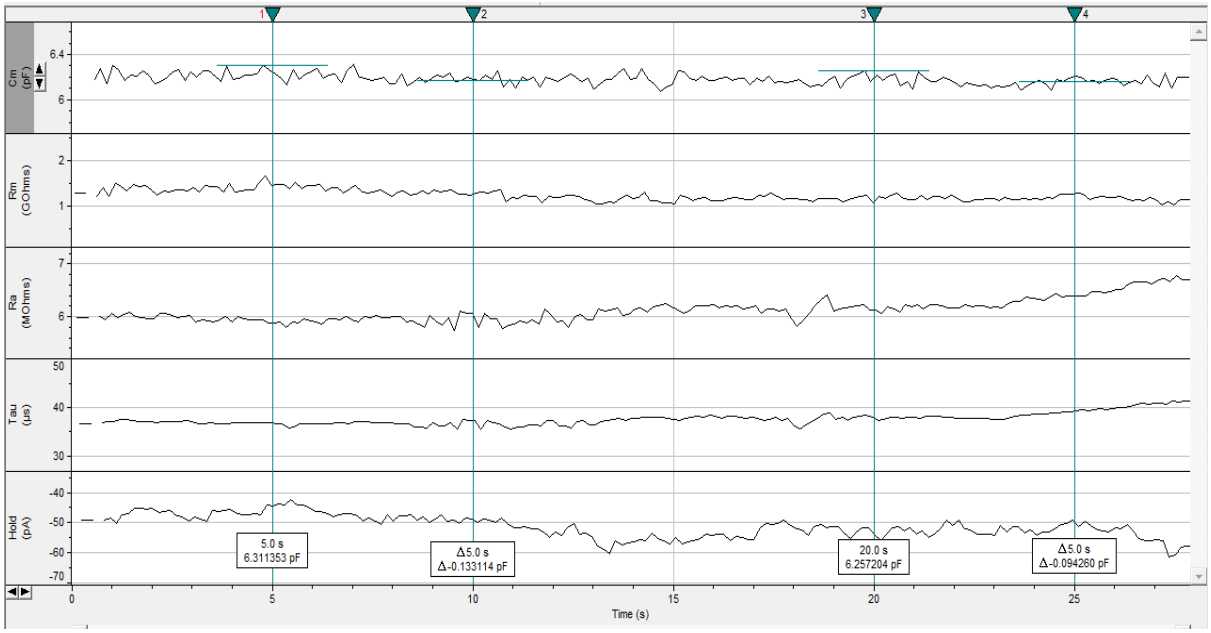


Figure 8: A representative membrane capacitance (Cm) tracing of bovine chondrocyte. The top trace of the membrane test function recordings shows the Cm recording in pF units.

II. Voltage Clamp Results

A. General characteristics of the bovine chondrocytes:

To study the general characteristics of the total current of the bovine chondrocytes, the initial voltage clamp recordings of the bovine chondrocytes perfused with ECF were taken. Two different stimulus waveform pulse protocols were used. The holding potential was -80mV in both protocols. In pulse protocol 1, the range was from -180mV to +105 mV, in 15mV steps which gave a total of 20 sweeps. In pulse protocol 2, the range was from -140mV to +80 mV, in 10mV steps which gave a total of 23 sweeps. Representative raw current tracings obtained from these two protocols are shown in Figure 9 and 10.

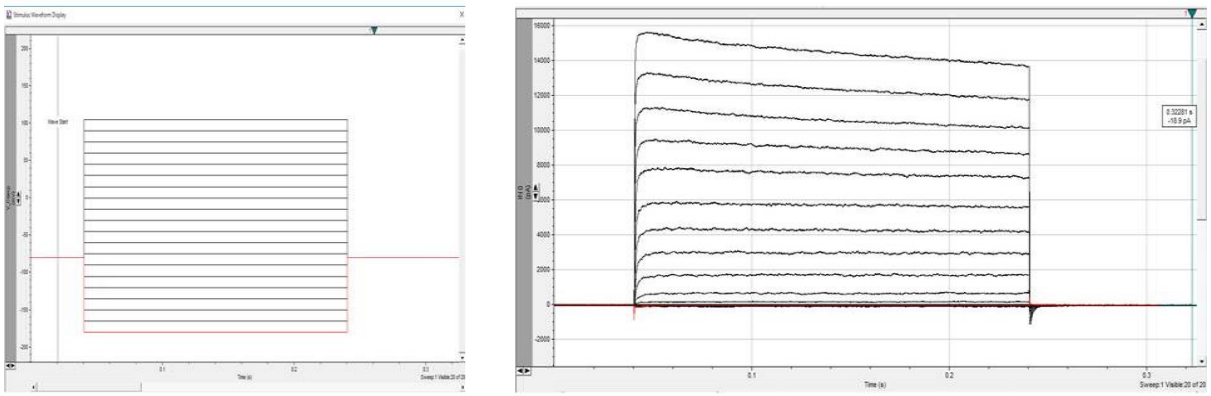


Figure 9: a representative raw current tracing from a chondrocyte using the stimulus waveform pulse protocol 1.

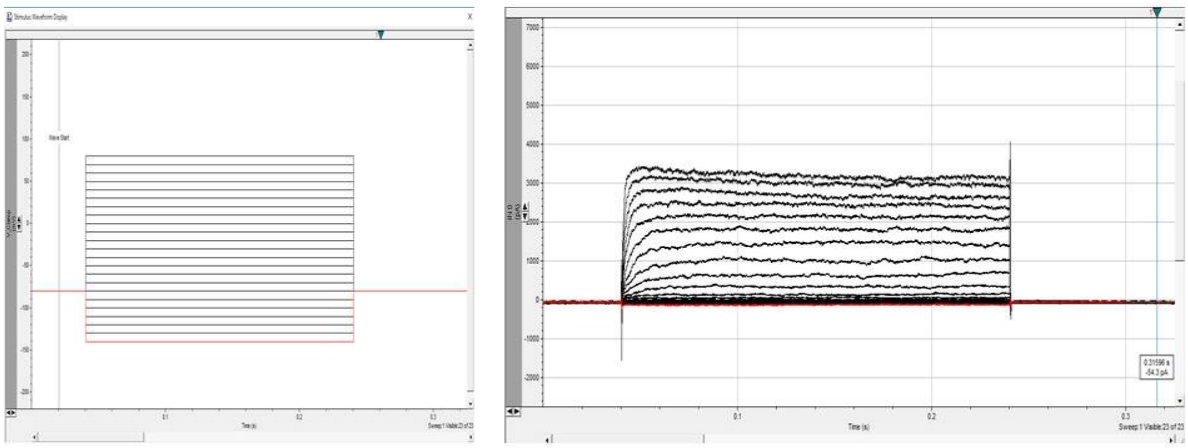


Figure 10: A representative raw current tracing from a chondrocyte using the stimulus waveform pulse protocol 2.

The Peak current was obtained for each pulse step from the current tracings segment (5 milli second after the step pulse was started till 100 milli seconds).The currents were measured in pico ampere units (pA). Peak current density was obtained by dividing the peak current with the membrane capacitance (Cm) of the cell. The unit of current density is in pA/pF.

A variation in current densities is seen in chondrocytes

Based on the magnitude of the current density (CD), the cells were divided into three groups –small CD (n=7), medium CD (n=5) and large CD (n=3). A similar variations in the current densities in goat articular chondrocytes were already shown in our lab (22). Mean ±SEM of the current density of these three groups at 60mV are shown in Figure 11.

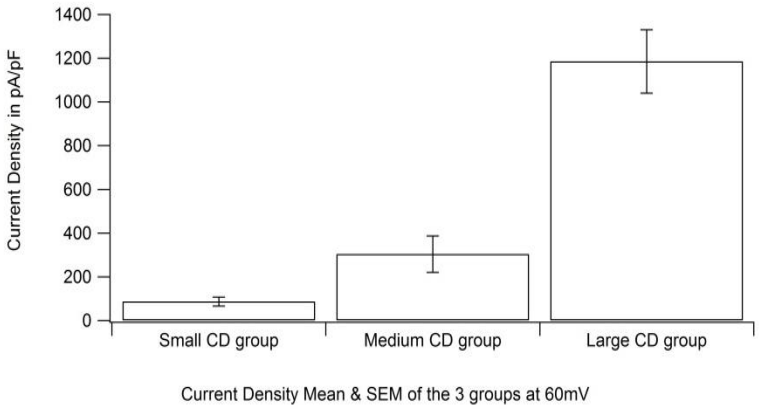


Figure 11: Current density mean ± SEM of the three groups at + 60mV step pulse .For small CD group n=7, for medium CD group n=5 and for large CD group n=3.

The mean peak current density was plotted against the step pulse voltages to obtain IV curves. The IV curves for the small, medium and large current density are shown in Figures 12,13,14 and 15.

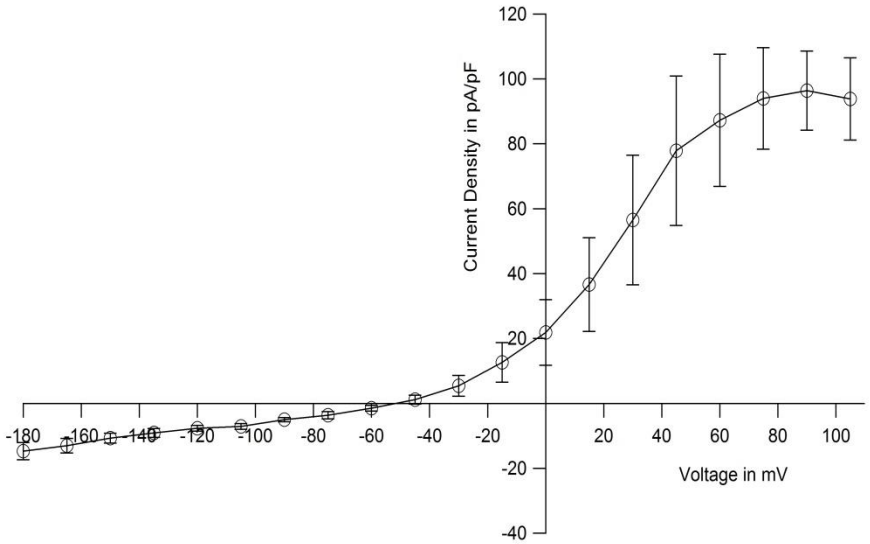


Figure 12: IV curve of chondrocytes that exhibit a small current density. Mean±SEM (n = 7).

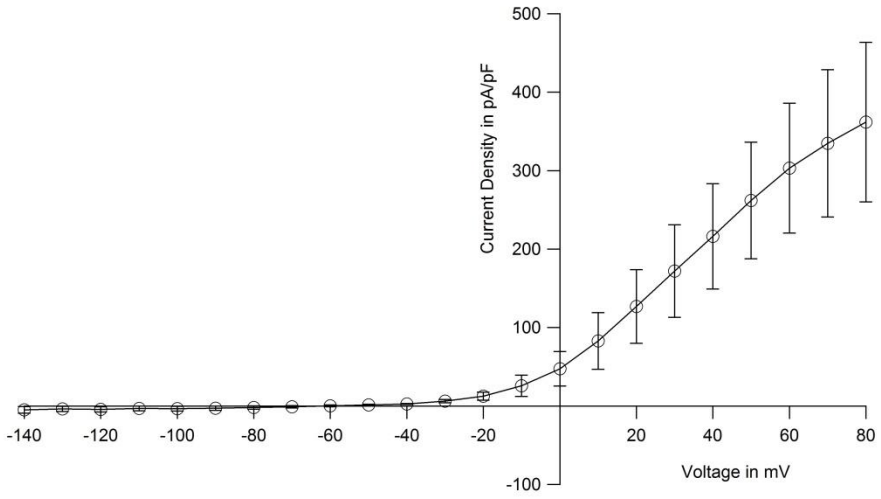


Figure 13: IV curve of chondrocytes that exhibit a medium current density. Mean±SEM (n = 5).

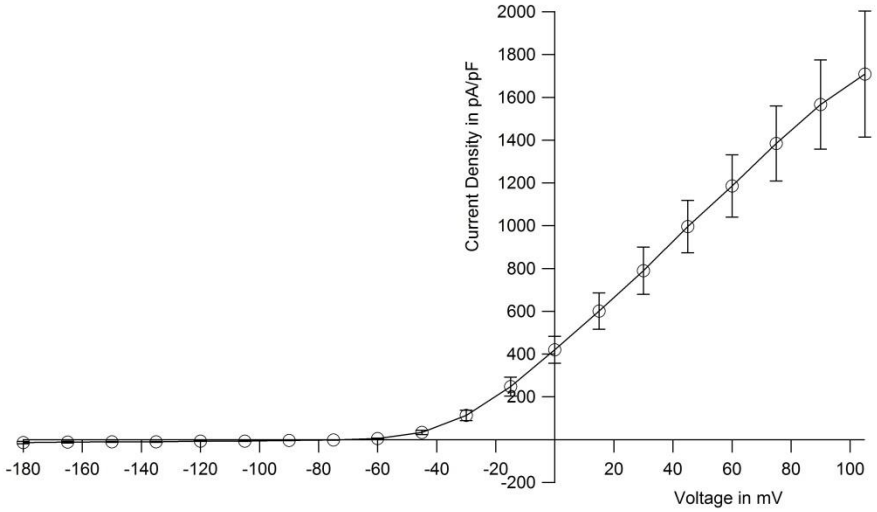


Figure 14: IV curve of chondrocytes that exhibit a large current density. Mean±SEM (n = 3).

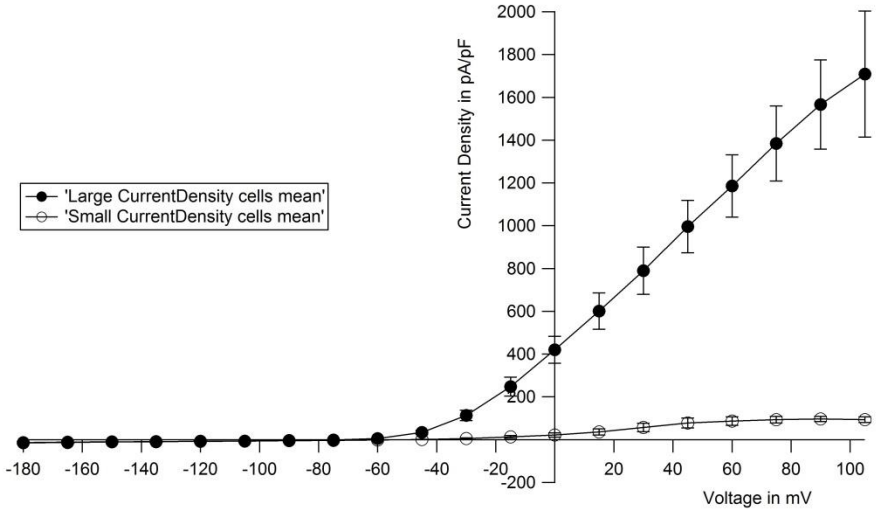


Figure 15: A comparison of the currents recorded from chondrocytes showing large (n=3) and small (n=7) current densities, Mean ± SEM.

Currents recorded showed a variation in inactivation.

Based on the inactivation property of the currents over time during the test step pulse period of 200 milli seconds, two types of currents were identified. For the majority of cells, the currents recorded did not inactivate over time and was constant over the entire test pulse. In a few cells (n=3) the outward currents recorded showed inactivation over time. This was seen at higher voltages. Representative raw tracings from both groups at 60mV and 105mV are shown in Figures 16 and 17. The mean current density IV curves obtained from three chondrocytes in each group are shown in Figure 18.

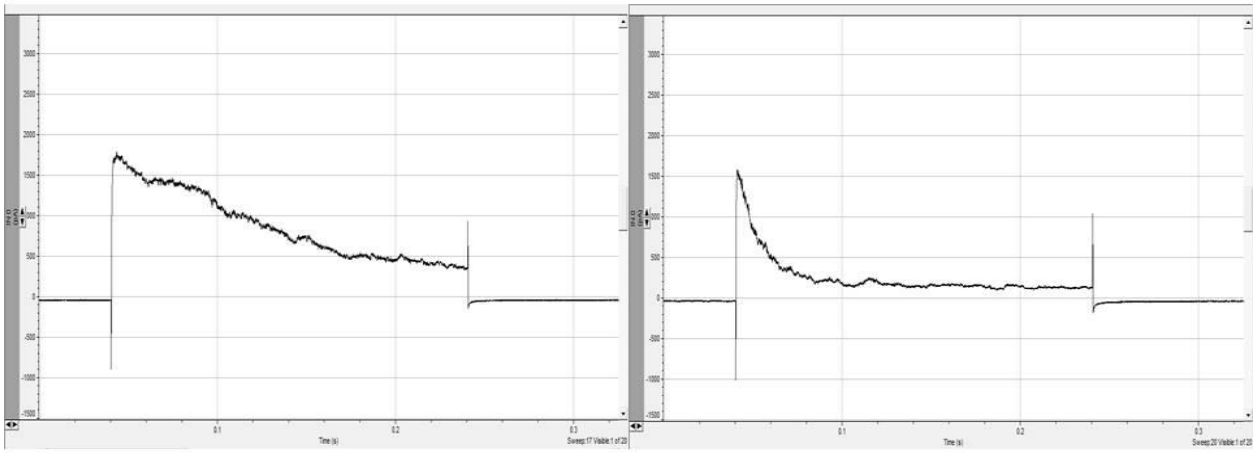


Figure 16: A representative raw current tracing from a bovine chondrocyte from which inactivating currents were recorded. Recordings are shown at 60mV and 105 mV step pulse.

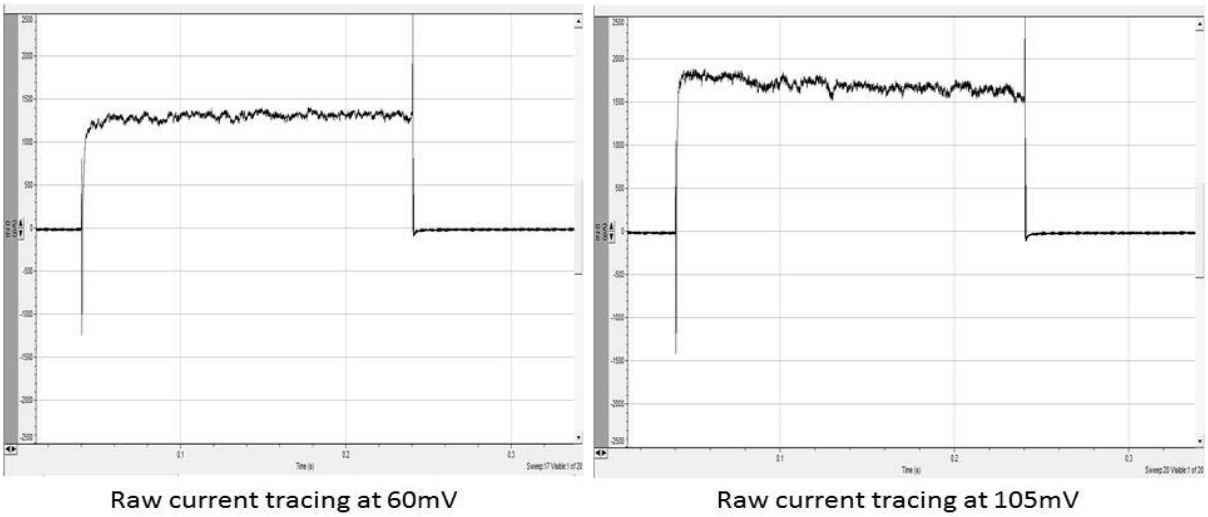


Figure 17: A representative raw current tracing from a bovine chondrocyte where currents that did not inactivate were recorded, the recordings taken at 60 mV and 105 mV step pulse.

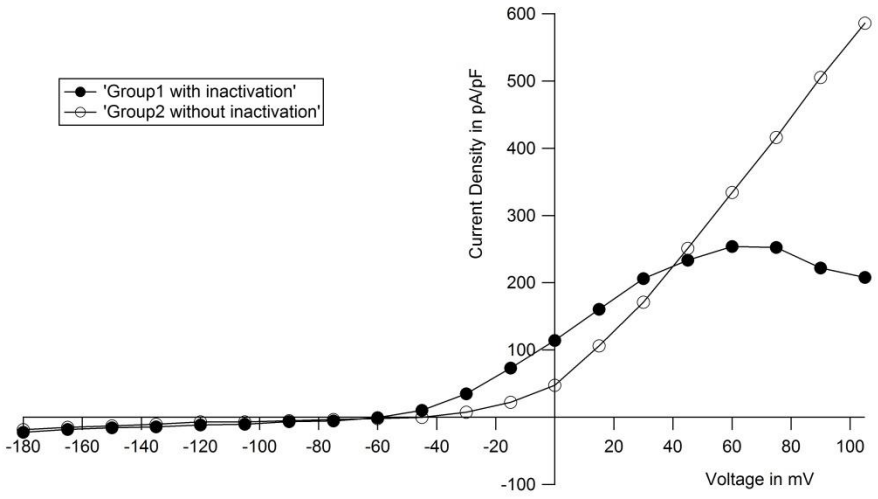


Figure 18: Current density vs voltage (IV) curves of two groups of currents recorded from bovine chondrocytes. The Group 1 showed inactivation of currents over time at higher voltages (n=3). Group 2 showed no inactivation of currents over time even at high voltages (n=3)

B. Tail Current results:

The reversal potential ($\mathbf{E_{rev}}$) was calculated from the voltage clamp recordings using the tail current protocol under ECF perfusion. A 5 milli second time segment just after the depolarization pre-pulse ended and in the test pulse initial period was chosen to get the mean current density. Tail current density mean and SEM was calculated from six bovine chondrocytes (shown in Table 4). Mean±SEM of the tail current density was plotted against the voltage and shown in Figure 19. From Figure 19, we can see the zero crossing was -60mV, and thus the $\mathbf{E_{rev}}$ was ~ -60mV.

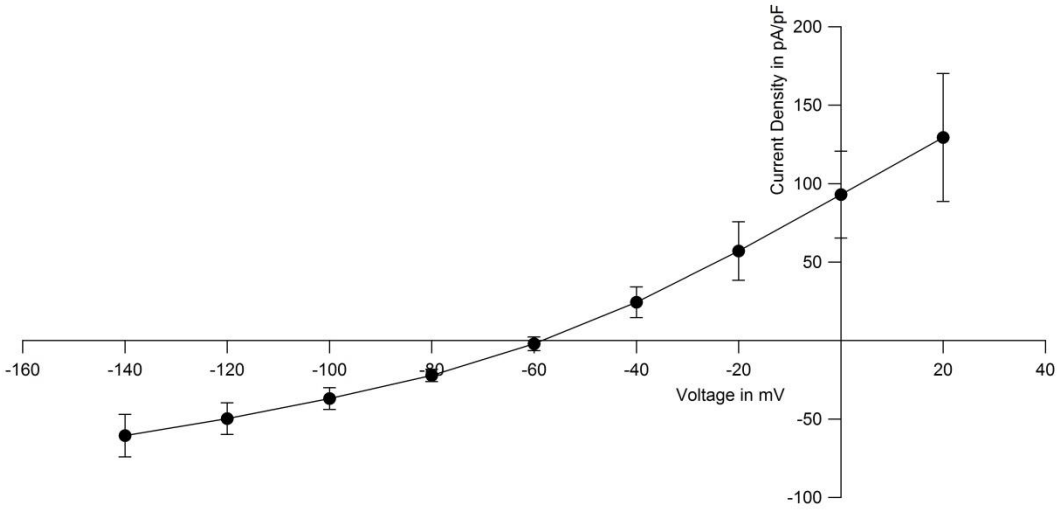


Figure 19: Tail current density IV curve: Mean±SEM of the tail current density were plotted against voltage (n=6). The currents were seen to reverse at -60 mV

We calculated the equilibrium potential for potassium ion ($\mathbf{E_k}$) using Nernst equation for equilibrium potential and found it to be equal to – 85.32mV. As the $\mathbf{E_{rev}}$ (- 60mV) was close to the calculated $\mathbf{E_k}$ (-85.32mV) ,it implies that the predominant ion species involved is potassium.

Voltage in mV	Mean Tail current Density (pA/pF)	SEM (pA/pF)
-140	-60.52	13.55
-120	-49.73	10.05
-100	-37.01	6.86
-80	-22.20	3.92
-60	-1.99	4.38
-40	24.42	9.73
-20	57.03	18.62
0	92.97	27.61
20	129.39	40.72

Table 4: Mean ± SEM of Tail current density (n=6).

The tail currents were also recorded under 10mM TEA perfusion for one bovine chondrocyte after recording under routine ECF perfusion. The recording is shown in shown in Figure 20. Under ECF perfusion the $\mathbf{E_{rev}}$ for this chondrocyte was -40mV and under 10mM TEA, $\mathbf{E_{rev}}$ shifted to near -10mV. This shows that the blockade of potassium channels with TEA affects the reversal potential of the currents recorded.

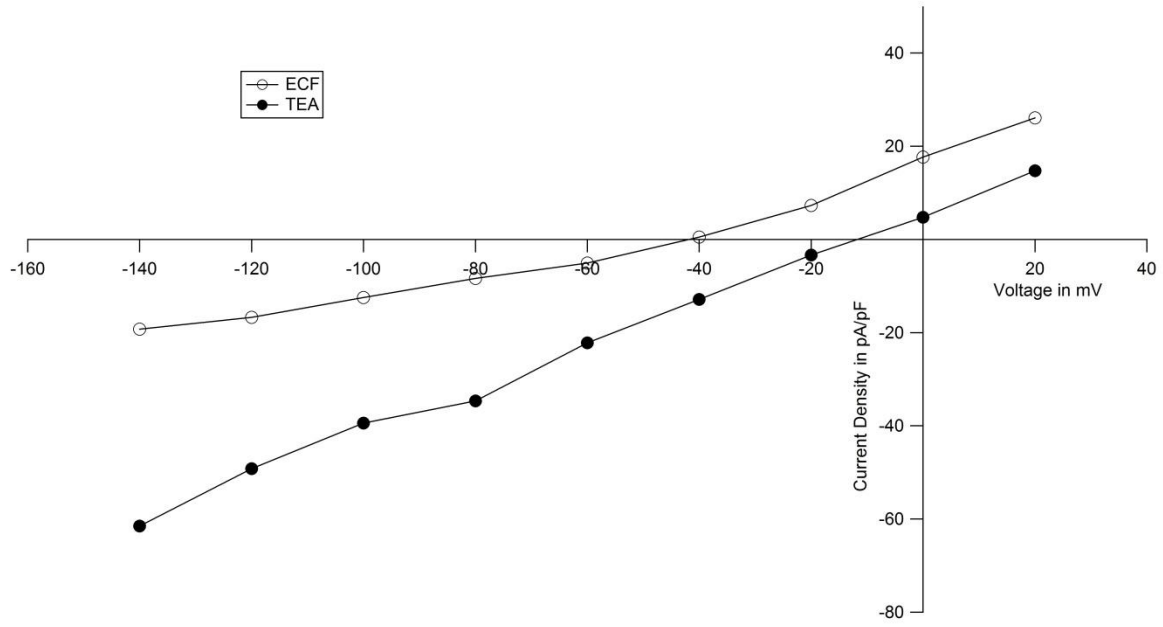


Figure 20: Effect of 10mM TEA on the tail current density of a bovine chondrocyte

C. Effect of TEA on currents.

10 mM TEA was used to block the potassium channels. The bovine chondrocytes were perfused with ECF and then with 10 mM TEA during the voltage clamp recording. The peak current density values at 0 mV and 60 mV were taken for calculating mean±SEM from seven chondrocytes and shown in the Table 5. Wilcoxon signed ranks test was used for statistical analysis. TEA reduced the currents in 6 out of

7 chondrocytes, whereas it increased the currents in one chondrocyte. Overall TEA reduced the currents. The reduction of currents were significant at 0mV and insignificant at 60mV (Figure 21 and 22). The current density IV curves with ECF and TEA perfusion are shown in Figure 23.

No	ECF-Peak current density(pA/pF) at 0 mV	TEA-Peak current density(pA/pF) at 0 mV		ECF-Peak current density(pA/pF) at 60 mV	TEA-Peak current density(pA/pF) at 60 mV
1	202.36	58.51		415.89	57.52
2	6.19	22.52		46.59	103.98
3	82.70	5.65		199.89	28.15
4	108.61	20.90		316.11	128.08
5	123.02	118.67		383.37	343.36
6	42.07	15.16		84.41	33.88
7	137.03	109.03		300.48	284.51
Mean	**100.28**	**50.06**		**249.53**	**139.93**
SEM	**24.35**	**17.63**		**54.23**	**47.37**
P	**.043**			**.091**	

Table 5: Mean ± SEM of ECF and TEA peak current densities at 0mV and 60mV. Wilcoxon Signed ranks test was used for statistical analysis.

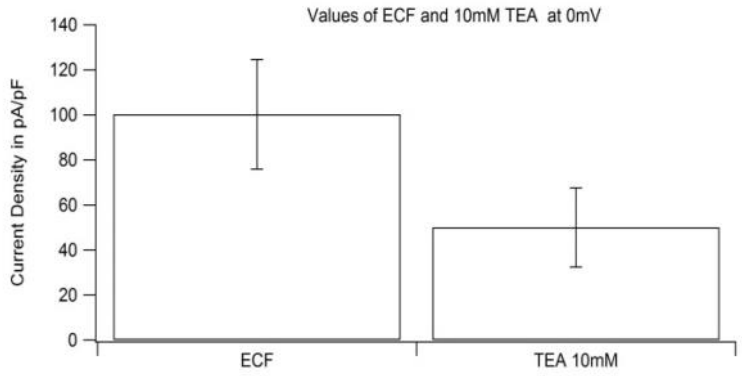


Figure 21: peak current density of chondrocytes recorded with ECF and TEA perfusion at 0 mV (n=7, Mean±SEM)

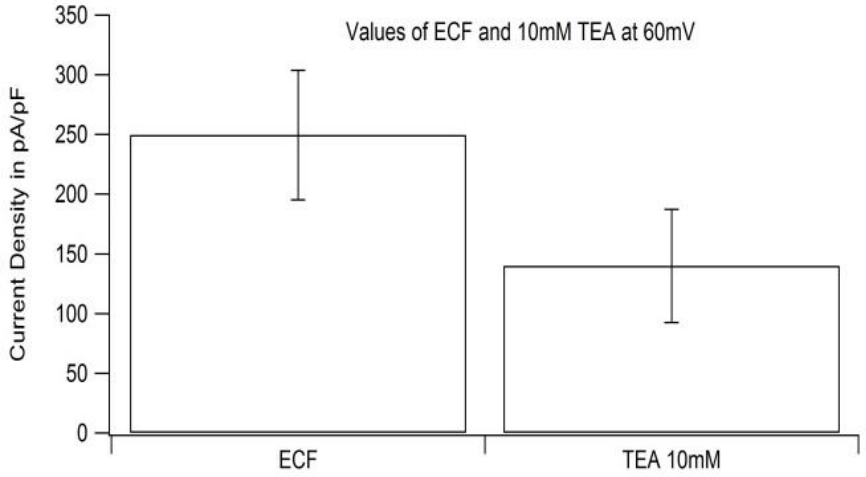


Figure 22: peak current density of chondrocytes recorded with ECF and TEA perfusion at +60 mV (n=7, Mean±SEM)

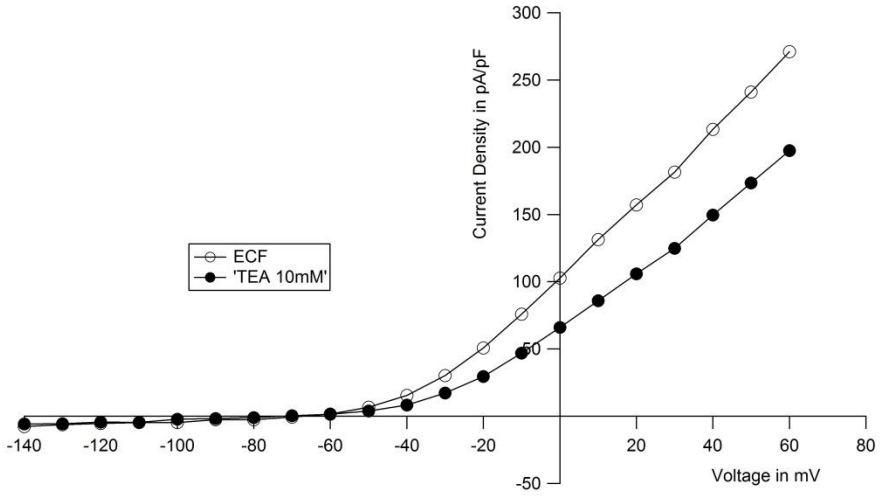


Figure 23: The effect of 10mM TEA on potassium currents in bovine articular chondrocytes (n=7, Mean).

D. The effect of H_2S:

NaHS was used as a H_2S donor in two different concentrations 500uM and 2mM. A 100mM NaHS stock solution was prepared by mixing the NaHS flakes in 10ml ECF and was sealed with parafilm, kept at -20^0 C and used either freshly or within two days. 500uM and 2mM NaHS were prepared by diluting the required volume of the stock solution in 10ml ECF and these solutions were prepared fresh on the day of patch clamp recordings.

The effect of 500uM NaHS:

Bovine chondrocytes were perfused with ECF and then 500uM NaHS during the voltage clamp recording. The peak current density values at 0 mV and 60 mV were taken from 14 bovine chondrocytes for calculating mean ± SEM as shown in the Table 6. Wilcoxon signed ranks test was used for statistical analysis, $P < 0.05$ was considered to be significant. Overall 500uM NaHS increased the peak current density slightly and the results were insignificant at both 0mV and 60mV. The Current density IV curves showing the effect of 500uM NaHS is shown in Figure 24. The effect at 0 mv is shown in Figure 25, n= 14, P=0.96. The effect at 60 mV is shown in Figure 26, n=14, P=0.3

Cell no.	ECF-Peak current density(pA/pF) at 0mV	500uM NaHS Peak current density (pA/pF) at 0mV		ECF-Peak current density(pA/pF) at 60mV	500uM NaHS Peak current density(pA/pF) at 60mV
1	544.80	513.82		1473.47	1434.41
2	349.59	468.43		1073.41	1150.07
3	77.07	102.58		510.34	536.96
4	10.08	10.77		57.22	18.80
5	231.29	212.45		446.95	457.64
6	5.41	5.39		57.18	54.09
7	6.41	4.36		30.40	20.04
8	12.82	47.52		96.98	117.49
9	79.10	78.29		184.59	146.10
10	31.75	49.98		130.21	107.11
11	179.39	203.31		533.69	545.08
12	4.25	75.03		20.31	232.00
13	15.73	17.59		115.95	174.47
14	26.95	164.01		458.88	772.73
Mean	112.48	139.54		370.68	411.93
SEM	43.35	43.96		115.71	117.92
P	.096			.300	

Table 6: Mean±SEM of peak current densities recorded with ECF and 500uM NaHS perfusion at 0mV and 60mV (n=14).

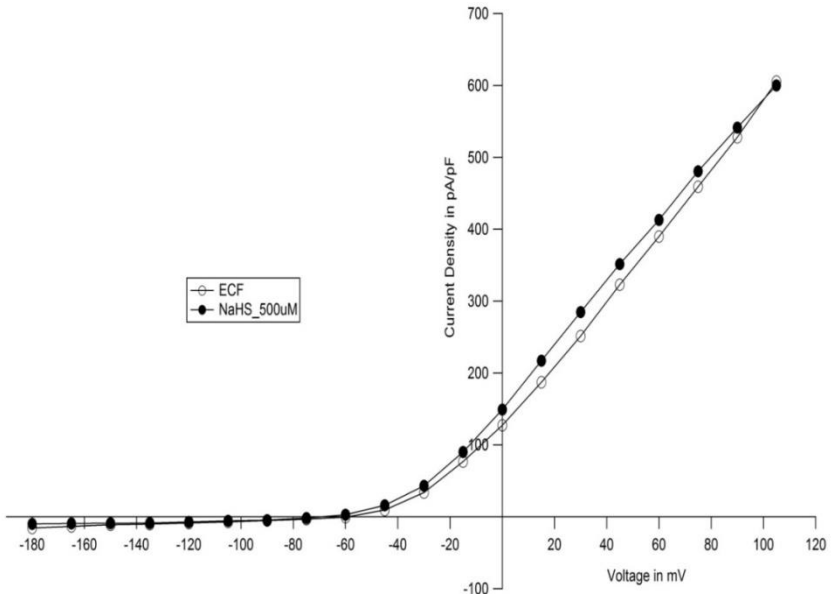


Figure 24: The effect 500uM NaHS on the current density of bovine chondrocytes, (n=14).

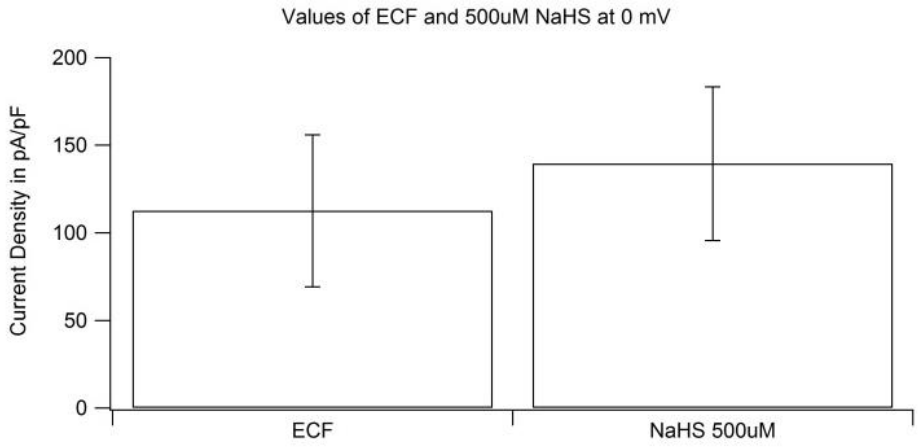


Figure 25: The effect of 500uM NaHS at 0mV on bovine chondrocytes, Mean ± SEM, (n=14).

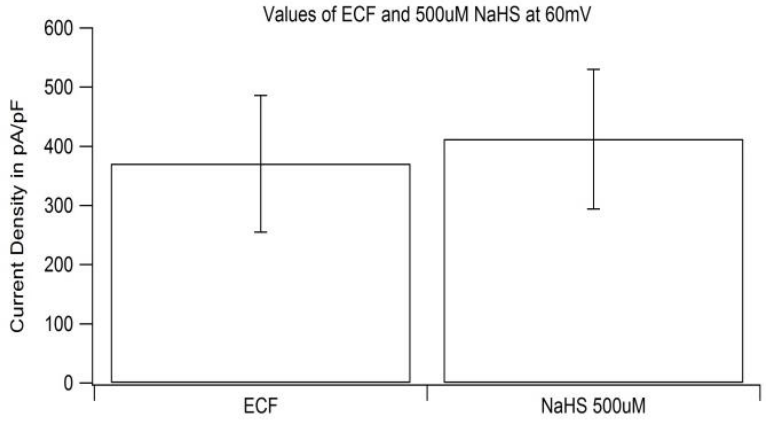


Figure 26: The effect of 500uM NaHS at 60mV on bovine chondrocytes, Mean ± SEM, (n = 14).

The effect of 2mM NaHS :

The bovine chondrocytes were perfused with ECF and then 2mM NaHS during the voltage clamp recording. The peak current density values at 0mV and 60 mV from 4 bovine chondrocytes were taken for calculating mean±SEM, as shown in Table 7. Overall, 2mM NaHS increased the peak current density. This effect however was not significant at 0 mV (P=0.14) or 60 mV (P=0.14). The mean increase was however larger than that seen at a 500 uM dose. The effect of 2mM NaHS is shown in Figures 27-29.

Cell no.	ECF-Peak current density(pA/pF) at 0mV	2mM NaHS Peak current density(pA/pF) at 0mV		ECF-Peak current density(pA/pF) at 60mV	2mM NaHS Peak current density (pA/pF)at 60mV
1	7.49	3.50		54.71	31.97
2	4.25	83.63		20.30	213.53
3	15.73	34.02		115.95	252.87
4	26.96	113.53		459.14	727.79
Mean	13.61	58.67		162.53	306.54
SEM	5.06	24.63		100.83	148.42
P	0.144			0.144	

Table 7: Mean±SEM of peak current densities recorded with ECF and 2mM NaHS perfusion at 0mV and 60mV (n=4).

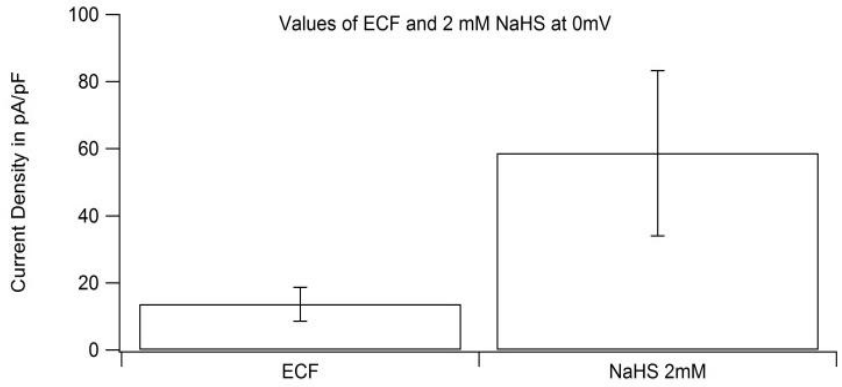


Figure 27: The effect of 2mM NaHS at 0mV on bovine chondrocytes, Mean ± SEM, (n=4).

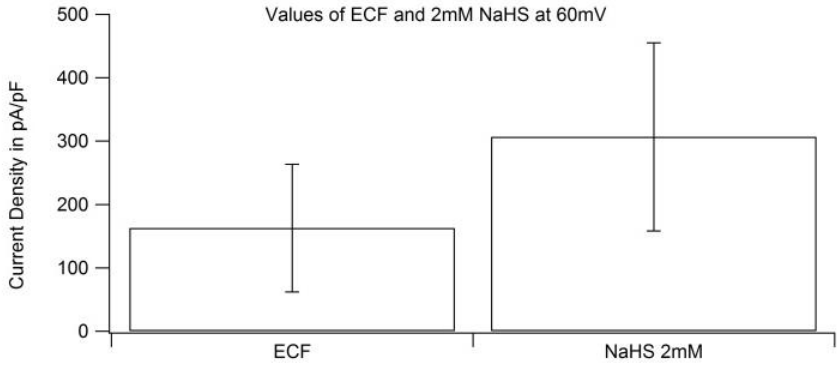


Figure 28: The effect of 2mM NaHS at 60mV on bovine chondrocytes, Mean ± SEM, (n=4).

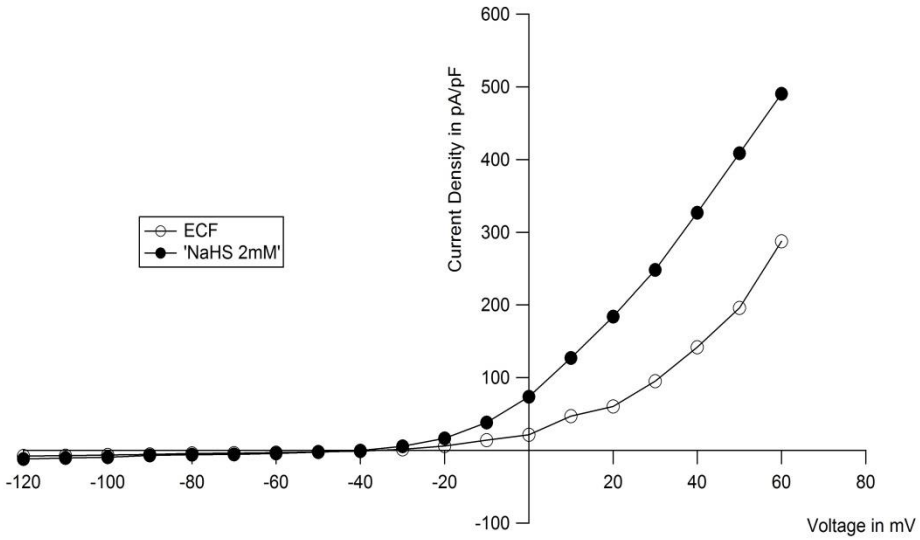


Figure 29: The effect of 2mM NaHS on the current density of bovine chondrocytes, (n=4)

E. Effect of L-Arginine:

L-Arginine was used as the substrate for nitric oxide synthases and its availability results in nitric oxide synthesis. Bovine chondrocytes were perfused with ECF and subsequently with 1mM L-Arginine during the voltage clamp recording. The peak current density values at 0mV and 60 mV from 2 bovine chondrocytes were taken for calculating mean±SEM as shown in the Table 8. A statistical test was not done as the data was insufficient. The trends were however noted. Overall, 1mM L-Arginine decreased the peak current density at 0mV and 60 mV as shown in Figures 30-32.

Cell no.	ECF-Peak current density(pA/pF) at 0mV	1mM L-Arginine Peak current density(pA/pF) at 0mV		ECF-Peak current density(pA/pF) at 60mV	1mM L-Arginine Peak current density(pA/pF) at 60mV
1	100.01	90.27		311.99	253.57
2	150.93	19.02		510.95	127.96
Mean	125.47	54.64		411.47	190.77
SEM	25.46	35.62		99.48	62.80

Table 8: Mean±SEM of peak current densities recorded with ECF and 1mM L-arginine perfusion at 0mV and 60mV (n=2).

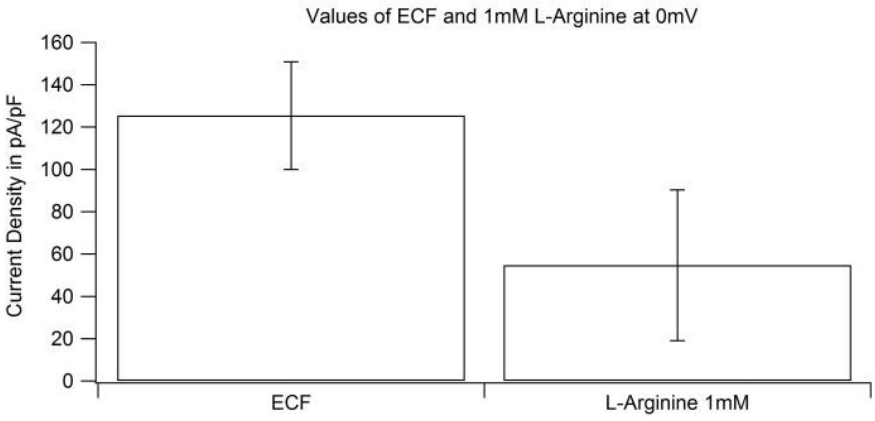


Figure 30: The effect of 1mM L-Arginine at 0mV on bovine chondrocytes, Mean ± SEM, (n=2).

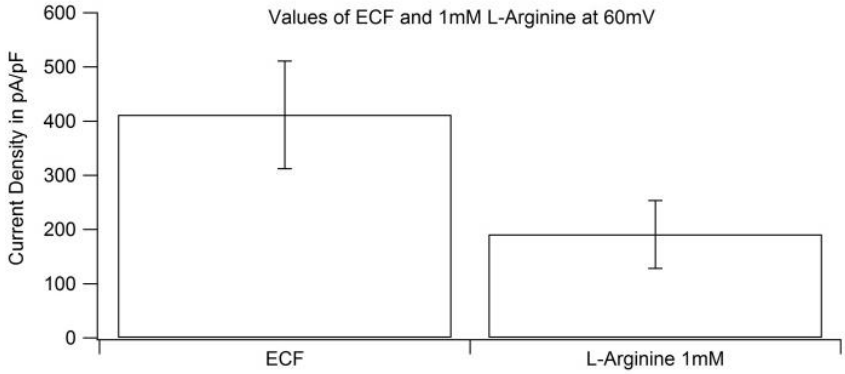


Figure 31: The effect of 1mM L-Arginine at 60mV on bovine chondrocytes, Mean ± SEM, (n=2).

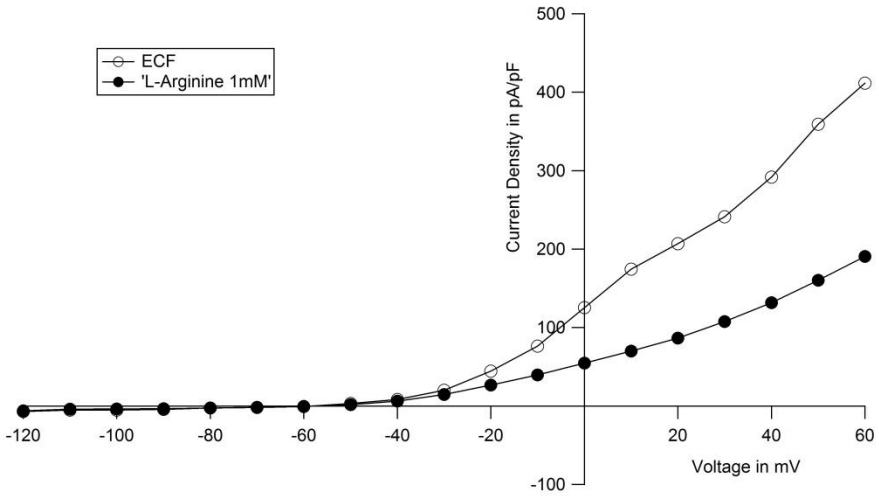


Figure 32: The effect of 1mM L-arginine on the current density of bovine chondrocytes, (n=2)

The effect of Lipopolysaccharide (LPS):

Lipopolysaccharide (LPS) was used in a concentration of 8ug/ml. LPS was used to mimic inflammatory conditions in bovine chondrocytes. Bovine chondrocytes were perfused with ECF and then perfused with LPS two times but at the same concentration (LPS 1st exposure and LPS 2nd exposure).

LPS 1st exposure effect

The peak current density values at 0mV and 60 mV from 4 bovine chondrocytes were taken for calculating mean±SEM, as shown in the Table 9. Overall, the first exposure effects of LPS showed a slightly increased peak current density at 0mV and slightly decreased peak current density at 60mV. This effect however was not significant, P = 0.273 and P= 0.465 respectively. The effect of the 1st exposure of LPS is shown in Figure 33.

Cell no.	ECF-Peak current density (pA/pF) at 0mV	1st exposure LPS Peak current density(pA/pF) at 0mV		ECF-Peak current density(pA/pF) at 60mV	1st exposure LPS Peak current density(pA/pF) at 60mV
1	35.33	46.41		196.83	246.74
2	28.54	93.10		465.01	408.22
3	380.64	330.45		944.52	775.43
4	286.45	361.79		1312.03	1324.52
Mean	182.74	207.94		729.60	688.73
SEM	89.17	80.60		248.20	239.05
P	.273			.465	

Table 9: Mean±SEM of peak current densities recorded with ECF perfusion and when perfused for the 1st exposure with LPS (8ug/mL), n=4.

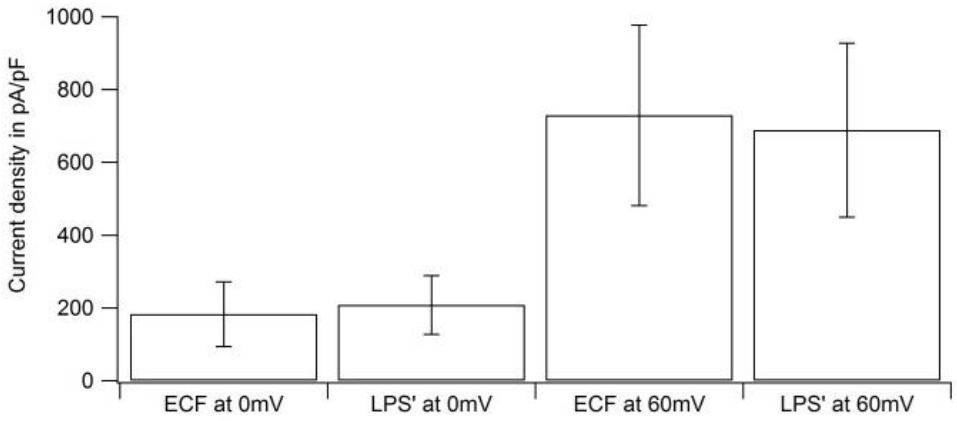


Figure 33: The effect of 1st exposure of LPS (8ug/mL) on current densities in bovine chondrocytes at 0mV and 60 mV, n= 4, Mean±SEM.

LPS 2nd exposure effects:

To assess the reaction to the cell when already exposed to LPS, a second exposure to 8ug/mL of LPS was performed. The peak current density values at 0mV and 60 mV from 3 bovine chondrocytes were taken for calculating mean±SEM, as shown in Table 10. Overall, the 2nd exposure effects of LPS showed a slightly increased peak current density at 0mV (P= 0.59) and a slightly decreased peak current density at 60mV (P= 0.593). This however was not found to be significant. The effects of the second exposure to LPS are shown in Figure 34. The combined effect of both the first and the second exposures to LPS is shown in Figure 35.

Cell no.	ECF-Peak current density(pA/pF) at 0mV	2nd exposure LPS Peak current density(pA/pF) at 0mV		ECF-Peak current density(pA/pF) at 60mV	2nd exposure LPS Peak current density(pA/pF) at 60mV
1	35.33	61.49		196.83	272.37
2	28.54	152.63		465.01	404.12
3	380.64	272.83		944.52	626.53
Mean	148.17	162.31		535.45	434.34
SEM	116.25	61.20		218.69	103.34
P	0.593			0.593	

Table 10: Mean±SEM of peak current densities recorded with ECF perfusion and when perfused for the 2nd exposure with LPS (8ug/mL), n=3.

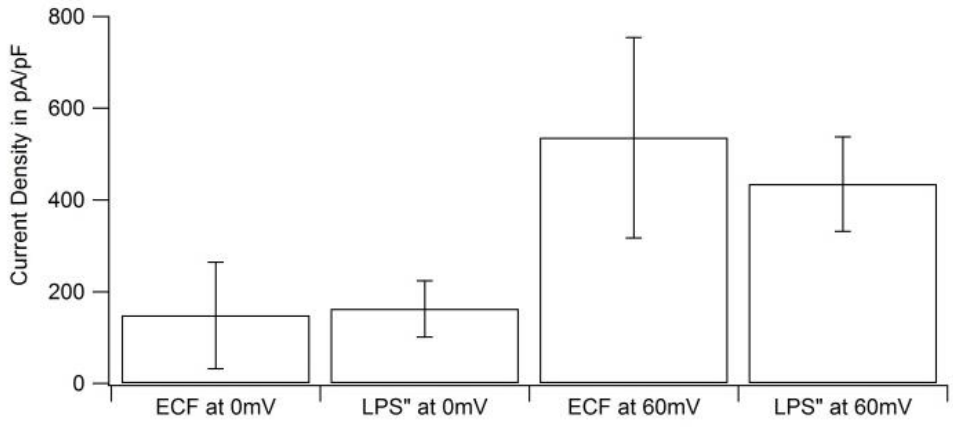


Figure 34: The effect of 2nd exposure of LPS (8ug/mL) on current densities in bovine chondrocytes at 0mV and 60 mV, n= 3, Mean±SEM.

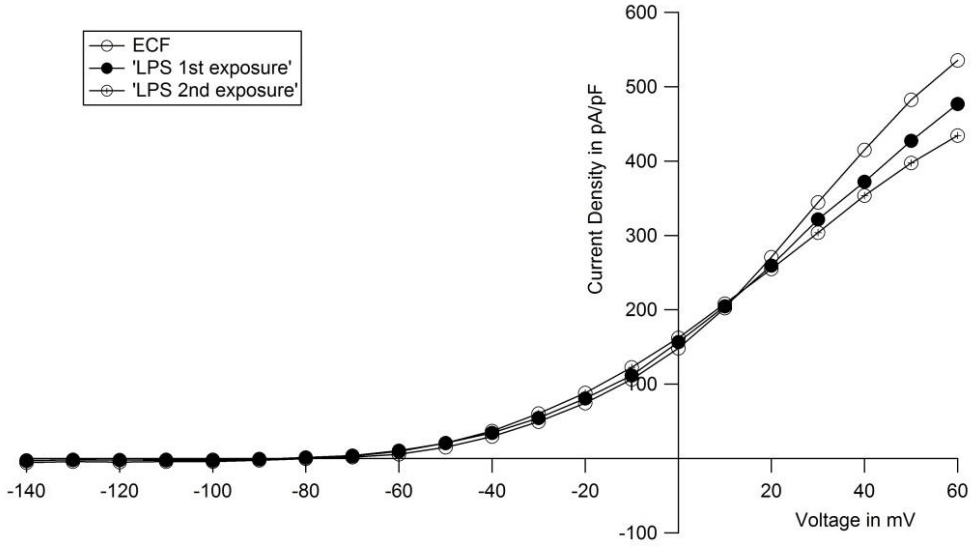


Figure 35: The effect of the 1st and 2nd exposure of LPS (8ug/mL) on the current densities of bovine articular chondrocytes (n = 3).

LPS + NaHS effects:

In one bovine chondrocyte, ECF perfusion was followed by LPS perfusion (three exposures). In all these three exposures, the current density increased. When perfused with LPS and NaHS subsequently, a decrease in the current density was seen. This phenomenon was recorded in only one cell and needs to be studied further. The Curve of these results are shown in Figure 36.

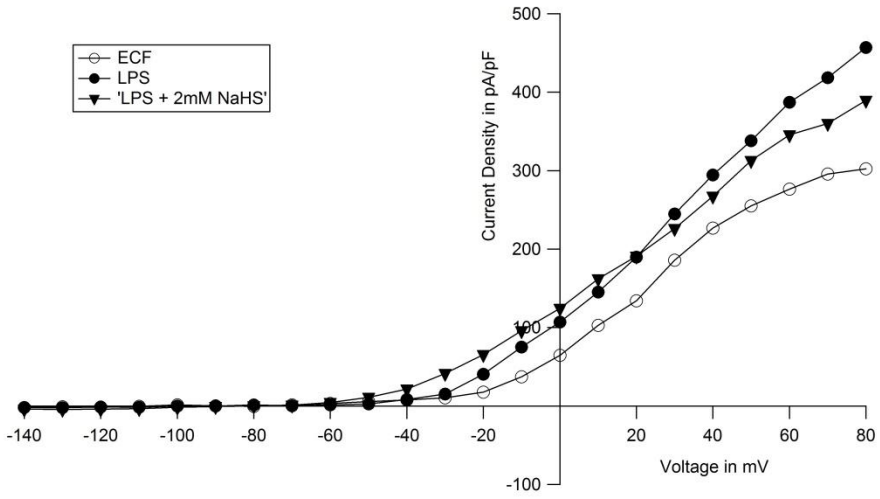


Figure 36: The effect of LPS + 2mM NaHS on a bovine articular chondrocyte. The current density with ECF perfusion, LPS perfusion (3rd exposure) and LPS + NaHS perfusion is shown.

III. CURRENT CLAMP RESULTS:

A. Resting Membrane potential (RMP) of the bovine chondrocytes:

The bovine chondrocytes membrane potential recorded under initial ECF perfusion immediately after the current clamp recordings were started was taken as the cells resting membrane potential (RMP). As mentioned earlier, an average trace from 10 sweeps was made. From the averaged trace, a 100 millisecond time segment was chosen just before the current injection pulse step and used to calculate the mean membrane potential. A representative raw tracing is shown in Figure 37. Mean ± SEM of the RMPs recorded from fourteen bovine chondrocytes is given in Table 11.

Cell no.	Resting Membrane potential
1	-56.6229
2	-13.9419
3	-27.9397
4	-29.0532
5	-13.6616
6	-12.3644
7	-22.9575
8	-37.6533
9	-21.5472
10	-19.5799
11	-21.7238
12	-41.3063
13	-4.22802
14	-17.1654
Mean	-24.2675
SEM	3.6337

Table 11: Mean ± SEM of the resting membrane potential of bovine articular chondrocytes (n=14).

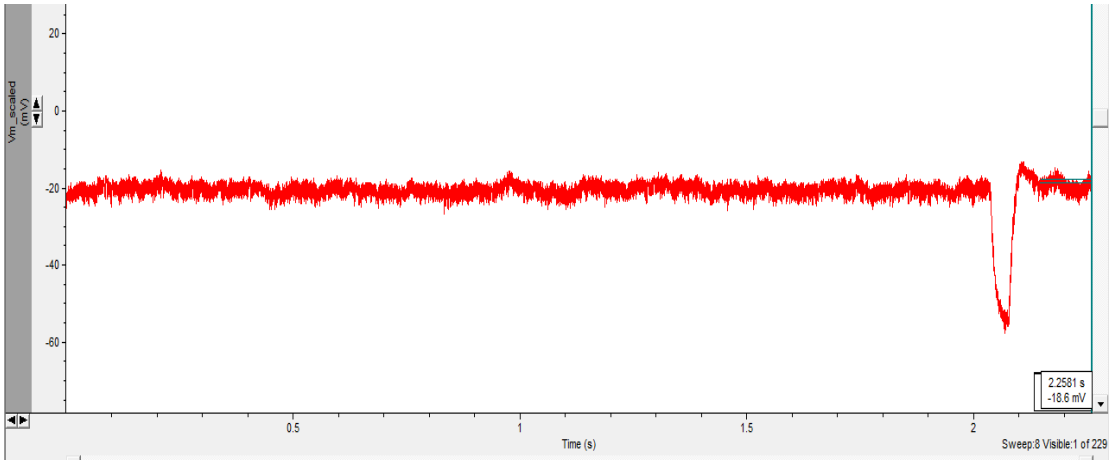


Figure 37: A representative raw tracing of membrane potential of a bovine chondrocyte in the current clamp mode. In this chondrocyte, the RMP is near -20mV and the membrane potential response to a short current injection pulse near the end of the sweep is seen.

B. Effect of 10mM TEA on the membrane potential of bovine chondrocytes:

10mM TEA was used as a potassium channel blocker. Mean and SEM were calculated from fourteen bovine chondrocytes as shown in Table 12. Out of 14 chondrocytes, 10mM TEA depolarized 9 cells and hyperpolarized 5 cells. Mean ± SEM of bovine chondrocyte membrane potential under ECF and 10mM TEA perfusion were -24.99 ± 3.67 mV and -20.2179 ± 3.2667 mV respectively. 10mM TEA depolarized the bovine chondrocyte membrane potential, however the effect seen was statistically insignificant (P = 0.221).

Cell no.	ECF: Membrane potential	10mM TEA: Membrane potential
1	-53.5508	-34.5349
2	-13.575	-10.3484
3	-31.5393	-5.29531
4	-43.5713	-22.1467
5	-38.9205	-35.6256
6	-27.0062	-24.8527
7	-19.2298	-26.3591
8	-5.22791	-8.87567
9	-25.12	-37.7881
10	-9.67896	-12.8592
11	-27.2602	-29.6725
12	-10.6719	-2.06766
13	-17.4994	-6.92977
14	-27.0345	-25.696
Mean	-24.9918	-20.2179
SEM	3.6771	3.2667
P	0.221	

Table 12: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with ECF and 10mM TEA (n= 14).

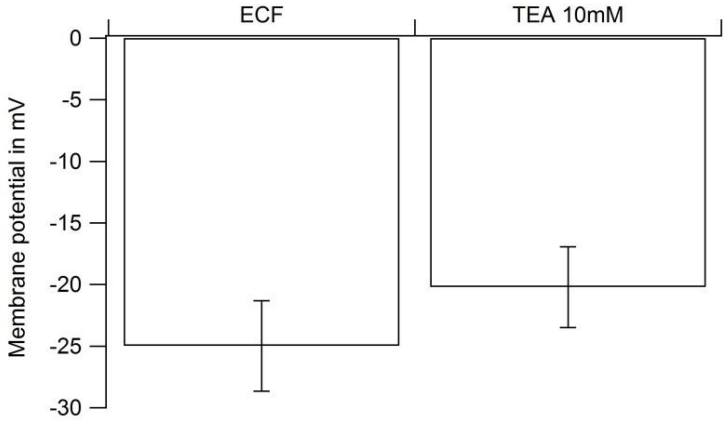


Figure 38: The effect of perfusion of 10mM TEA on the membrane potential of bovine articular chondrocytes, mean ± SEM (n = 14).

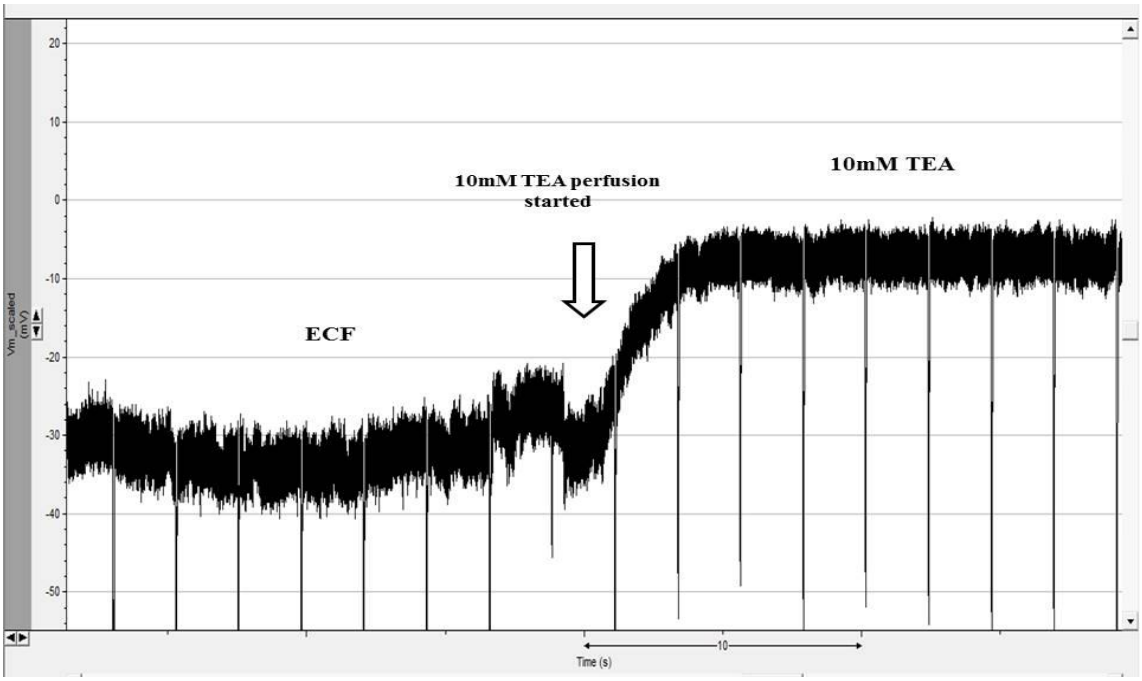


Figure 39: A representative raw membrane potential tracing showing the depolarizing effect of 10mM TEA on the membrane potential of a bovine articular chondrocyte.

C. The effect of NaHS on the membrane potential of bovine articular chondrocytes:

500uM NaHS was used as the H_2S donor. Mean and SEM were calculated from six bovine chondrocytes as shown in Table 13. Mean ± SEM of bovine chondrocyte membrane potential under ECF and 500uM NaHS perfusion were -31.10 ± 7.52 mV and -29.19 ± 8.36 mV respectively. 500uM NaHS slightly depolarized the bovine chondrocyte membrane potential, however this effect was not statistically significant (P = 0.6).

Cell no.	ECF: Membrane potential	500uM NaHS: Membrane potential
1	-56.6379	-57.1798
2	-15.5508	-12.1105
3	-13.8058	-22.4642
4	-27.8436	-29.1057
5	-21.5915	-5.1236
6	-51.1952	-49.2014
Mean	-31.1041	-29.1975
SEM	7.5233	8.3669
P	0.6	

Table 13: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with ECF and 500uM NaHS (n= 6).

The effect of 500 uM NaHS on bovine cartilage is shown in Figure 40.

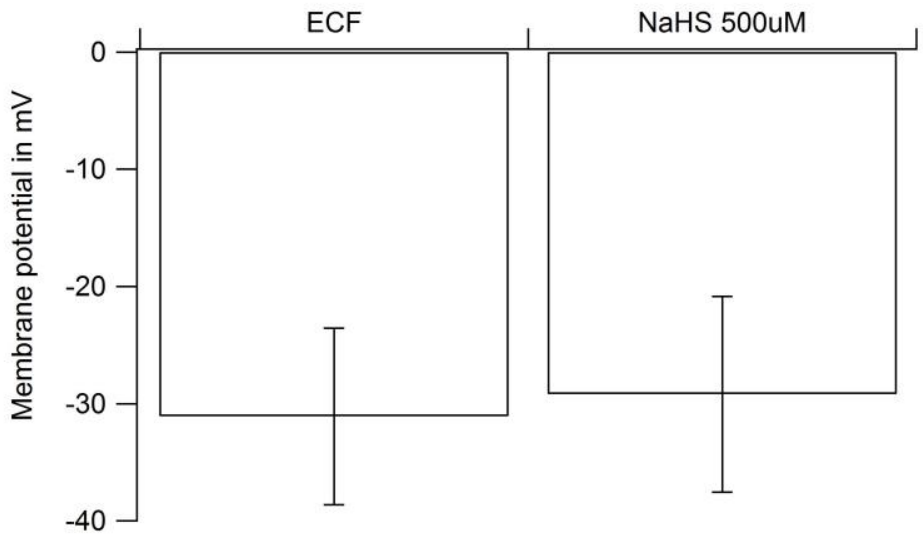


Figure 40: The effect of perfusion of 500uM NaHS on the membrane potential of bovine articular chondrocytes, mean ± SEM (n = 6).

Effect of 2mM NaHS on the membrane potential of bovine chondrocytes:

2mM NaHS was used as the H_2S donor. Mean and SEM were calculated from six bovine chondrocytes as shown in Table 14. Mean ± SEM of bovine chondrocyte membrane potential under ECF and 2mM NaHS perfusion were -33.55 ± 6.83mV and -30.43 ± 7.80 mV respectively. 2mM NaHS slightly depolarized the bovine chondrocyte membrane potential. The effect was however not statistically significant (P = 0.173).The depolarizing effect of NaHS was more at 2mM concentration than at 500uM concentration. The effect of 2mM NaHS is seen in figure 41

Cell no.	ECF: Membrane potential	2mM NaHS: Membrane potential
1	-57.9071	-52.1042
2	-19.576	-11.2757
3	-21.9292	-17.5419
4	-41.3853	-46.1339
5	-44.3157	-44.5515
6	-16.2254	-10.9761
Mean	-33.5564	-30.4305
SEM	6.8337	7.8043
P	0.173	

Table 14: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with ECF and 2mM NaHS (n= 6).

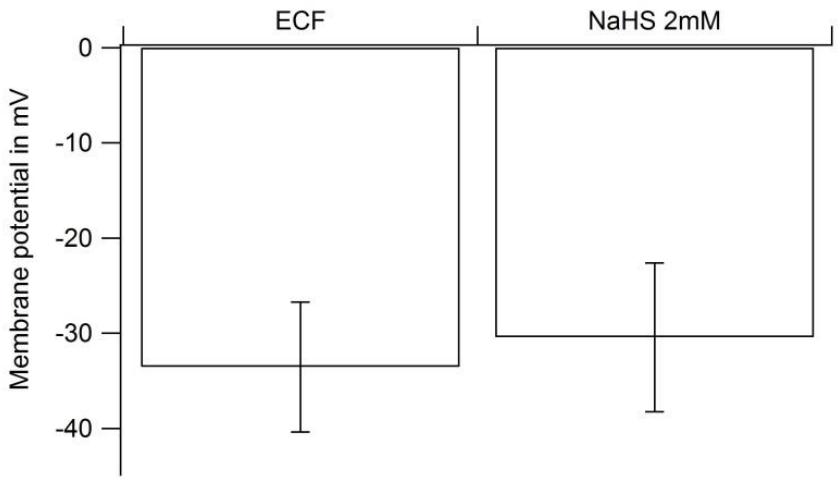


Figure 41: The effect of perfusion of 2mM NaHS on the membrane potential of bovine articular chondrocytes, mean ± SEM (n = 6).

D. The effect of 1mM L-Arginine on the membrane potential of bovine chondrocytes:

1mM L-Arginine was used as the substrate for nitric oxide synthases which results in nitric oxide synthesis. Mean and SEM were calculated from nine bovine chondrocytes as shown in Table 15. Mean ± SEM of bovine chondrocyte membrane potential under ECF and 1mM L-Arginine perfusion were -23.98±6.43mV and -27.83±5.01 mV respectively. 1mM L-Arginine hyperpolarized the bovine chondrocyte membrane potential, however this effect was not statistically significant (P =0.37). The effect of L-Arginine on the membrane potential of chondrocytes is seen in Figure 42.

Cell no.	ECF: Membrane potential	1mM L-Arginine: Membrane potential
1	-3.41203	-3.32743
2	-53.1018	-45.0912
3	-10.0209	-15.3884
4	-13.1168	-13.5933
5	-48.0221	-45.4318
6	-41.2707	-43.3451
7	-4.23398	-26.9598
8	-29.0532	-28.7611
9	-13.6616	-28.6443
Mean	-23.9881	-27.8380
SEM	6.4367	5.0119
P	0.37	

Table 15: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with ECF and 1mM L-Arginine, n= 9

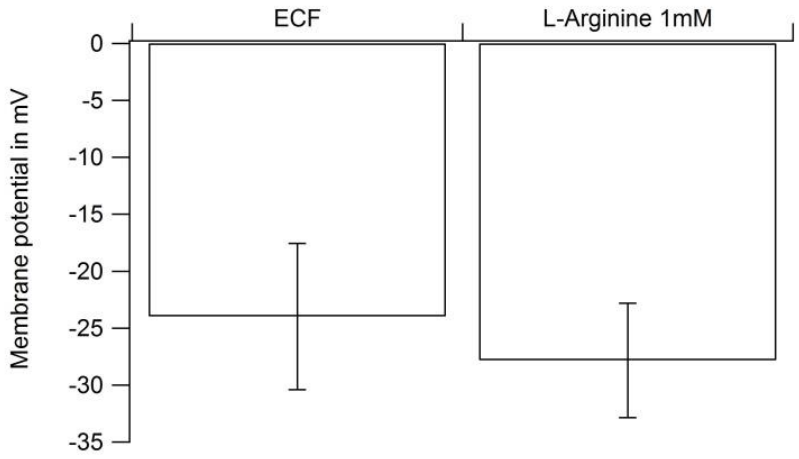


Figure 42: The effect of perfusion of 1mM L-Arginine on the membrane potential of bovine articular chondrocytes, mean ± SEM (n = 9).

E. The effect of 1mM L-Arginine + 2mM NaHS on the membrane potential of bovine chondrocytes:

To assess the interaction between H_2S and nitric oxide both 1mM L-Arginine and 2mM NaHS were perfused simultaneously. Mean and SEM were calculated from five bovine chondrocytes as shown in Table16. Mean ± SEM of bovine chondrocyte membrane potential under ECF and 1mM L-Arginine + 2mM NaHS perfusion were -36.49 ± 6.97mV and -33.90 ± 5.59mV respectively. 1mM L-Arginine + 2mM NaHS had a slight depolarizing effect on bovine chondrocyte membrane potential. This effect was however not statistically significant (P =0.5). The effect of this combined perfusion is seen in Figure 43

Cell no.	ECF: Membrane potential	1mM L-Arginine + 2mM NaHS: Membrane potential
1	-51.1952	-51.7116
2	-28.2755	-25.1168
3	-13.1168	-23.1838
4	-45.591	-42.3187
5	-44.3157	-27.175
Mean	-36.4988	-33.9011
SEM	6.9788	5.5916
P	0.5	

Table 16: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with ECF and a combination of 1mM L-Arginine + 2mM NaHS, (n= 5).

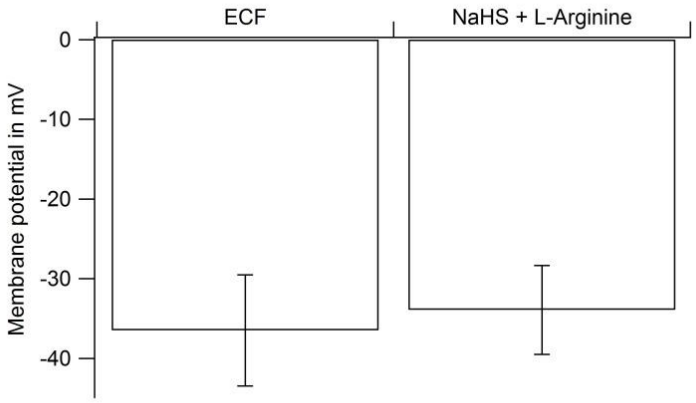


Figure 43: The effect of perfusion of 1mM L-Arginine+2mM NaHS on the membrane potential of bovine articular chondrocytes, mean ± SEM (n = 5).

F. The effect of 2mM LNNA on the membrane potential of bovine chondrocytes:

2mM LNNA was used as an inhibitor of Nitric oxide synthases which results in an inhibition of nitric oxide synthesis. Mean and SEM were calculated from three bovine chondrocytes as shown in Table 17. Mean ± SEM of bovine chondrocyte membrane potential under ECF and 2mM LNNA perfusion were -30.03 ± 9.80mV and 0.53 ± 1.86 mV respectively. 2mM LNNA depolarized the bovine chondrocyte membrane to a large extent (Figure 44), however this effect was not statistically significant (P =0.109).

Cell no.	ECF: Membrane potential	2mM LNNA: Membrane potential
1	-13.4076	3.82163
2	-29.3375	-2.64145
3	-47.3474	0.432382
Mean	-30.0308	0.5375
SEM	9.8037	1.8664
P	0.109	

Table 17: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with LNNA (2mM) and ECF (n= 3).

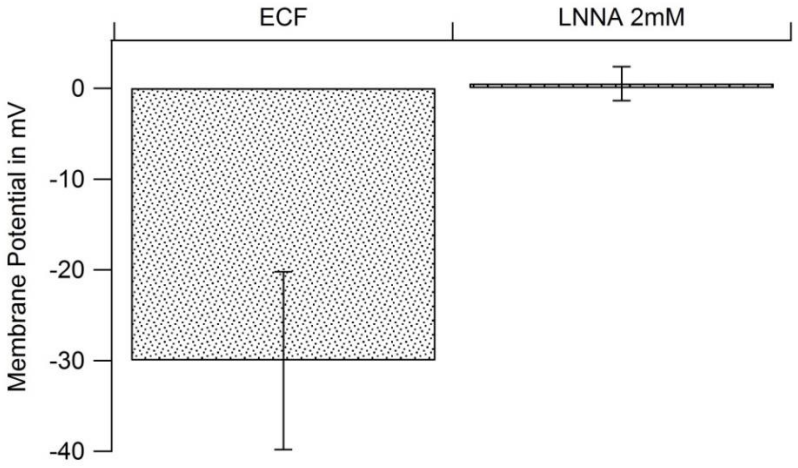


Figure 44: The effect of perfusion of 2mM LNNA on the membrane potential of bovine articular chondrocytes, mean ± SEM (n = 3).

G. The effect of LPS (8ug/ml) on the membrane potential of bovine chondrocytes:

Lipopolysaccharide (LPS) was used to mimic inflammatory conditions. LPS was used in the concentration of 8ug/ml. The effect of LPS on the bovine chondrocyte membrane potential during initial (1^{st}) and later (2^{nd} or 3^{rd}) exposure perfusions was studied. Mean and SEM were calculated from 2 bovine chondrocytes for both initial and later exposure as shown in Table 18 and 19. In the LPS later exposure experiments, after the LPS perfusion, 2mM NaHS+LPS was perfused to study the interaction between H_2S and LPS. Mean ± SEM of bovine chondrocyte membrane potential under ECF and initial exposure LPS perfusion were -30.30 ± 7.34 mV and -36.87 ± 2.99 mV respectively. Initial exposure LPS perfusion hyperpolarized the bovine chondrocyte membrane potential.

Mean ± SEM of bovine chondrocyte membrane potential under ECF and later exposure LPS, and LPS+2mM NaHS perfusion were -28.77±2.21 mV, -25.87±2.06 mV and -27.0±2.13 mV respectively. There were no significant changes seen with these interventions.

Cell no.	ECF: Membrane potential	LPS initial exposure: Membrane potential
1	-22.9575	-33.8825
2	-37.6533	-39.8676
Mean	-30.3054	-36.8750
SEM	7.3479	2.9925

Table 18: Mean ± SEM of the membrane potential of bovine chondrocytes when perfused with ECF and an initial exposure LPS (8ug/mL), n= 2.

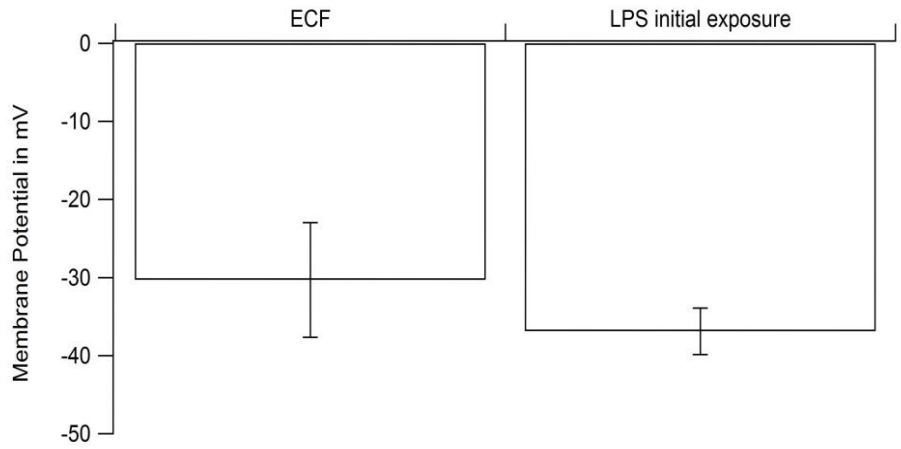


Figure 45: The effect of perfusion of 8ug/ml LPS (initial exposure) and perfusion of ECF on the membrane potential of chondrocytes, n=2.

Cell no.	ECF: Membrane potential	LPS later exposure: Membrane potential	LPS + 2mM NaHS: Membrane potential
1	-26.5622	-23.8079	-24.8665
2	-30.9836	-27.9334	-29.1344
Mean	-28.7729	-25.8706	-27.0004
SEM	2.2107	2.0627	2.1339

Table 19: Mean±SEM of the membrane potential of bovine chondrocytes perfused with ECF and a later exposure of LPS (8ug/mL), as well as LPS+NaHS, n= 2.

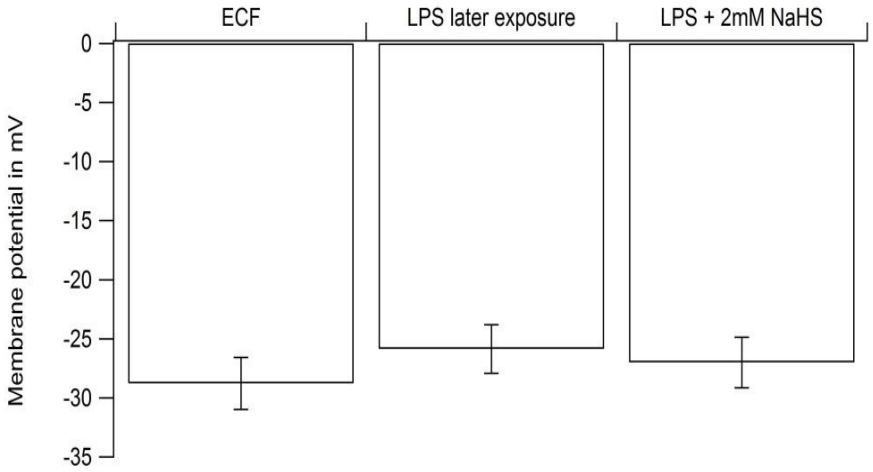


Figure 46: The effect of perfusion of 8ug/ml LPS (later exposure), perfusion of ECF as well as perfusion of LPS+NaHS on the membrane potential of chondrocytes, n=2.

DISCUSSION

DISCUSSION:

This study attempted to understand the role of H_2S and NO on the electrophysiology of bovine articular chondrocytes in normal and inflammatory conditions. Lipopolysaccharide (LPS) was used to mimic the inflammatory condition in bovine articular chondrocytes.

We tested the effects of NO using L-Arginine (a nitric oxide synthase substrate) separately and wanted to study the interaction between NO and H_2S on the bovine articular chondrocyte electrophysiology.

Based on previous literature, a large component of delayed rectifier currents was expected in articular chondrocytes. The current recorded was similar to the profile of currents passing through delayed rectifier potassium channels. These currents were inhibited by 10mM TEA.

The mean membrane potential of the bovine articular chondrocytes was -24.26 mV, which is consistent with the values reported in previous studies. The chondrocyte resting membrane potential is less negative than other cells. 10mM TEA depolarized the chondrocyte membrane potential demonstrating that a TEA sensitive potassium conductance plays a role in the maintenance of the chondrocyte resting membrane potential.

A hyperpolarization of the membrane potential was expected on the addition of H_2S and NO. This was expected under non-inflammatory conditions. The addition of H_2S

however caused a small depolarization. The addition of L-arginine (a substrate for nitric oxide synthases, leading to NO synthesis) caused a mild hyperpolarization as expected. However the changes produced were not statistically significant.

Similarly NO and H_2S were expected to increase the potassium currents and cause potassium efflux. Our experiments showed that NaHS increased potassium currents. L-arginine on the other hand decreased the potassium currents. These results were also not statistically significant.

Although the results are not significant, which may be due to the small numbers recorded, a deviation from what was expected could be due to the fact that chondrocytes in culture may behave differently from native chondrocytes.

This study also aimed to document if H_2S and NO acted synergistically or antagonistically. The results obtained indicate a trend of antagonistic action.

A large depolarization of the chondrocyte membrane potential was seen on the addition of LNNA. This indicates that nitric oxide may play a significant role in the regulation of the chondrocyte membrane potential. Although the effect was large, this effect was not statistically significant as the number of cells that were recorded for intervention was small. This effect however has to be studied in further detail.

Lipopolysaccharide showed a biphasic membrane potential response. The lipopolysaccharide initial exposure produced a hyperpolarization of the chondrocyte membrane potential, while a subsequent later exposure of lipopolysaccharide produced a depolarization of the chondrocyte membrane potential. Lipopolysaccharide exposure also showed varied current responses where 3 out of 4

bovine chondrocytes showed a decrease in potassium currents. In one cell an increase in currents were observed. These results were not significant, however the trend needs to be investigated further.

This study also aimed to analyze how chondrocytes respond to H_2S and Nitric oxide in inflammatory conditions. This was possible in two cells. These cells showed indications that a later LPS exposure produced a depolarization which was reversed when 2mM NaHS was perfused concurrently with LPS. Further experiments on these lines will be required to identify if these trends are significant.

Although many results in this study are not significant, this study to the best of our knowledge is the first one to test the effects of H_2S, nitric oxide and LPS on bovine articular chondrocyte electrophysiology. The trends obtained also suggest the need for further studies with larger sample size and with the use of specific inhibitors and donors of H2S and nitric oxide.

SUMMARY

SUMMARY:

1. Bovine articular chondrocytes demonstrated the presence of large outward currents that could be inhibited by TEA, whose IV profile suggested the presence of delayed rectifier potassium channels. These currents did not inactivate over time. The current density varies widely from cell to cell.
2. The recorded reversal potential was -60mV which is near the calculated equilibrium potential for the potassium (-85.32 mV). The reversal potential was also shifted to the right by TEA once again suggesting the involvement of potassium channels. The deviation of the reversal potential from the calculated value suggests that other ionic conductance may also be active in these situations.
3. The mean resting membrane potential of bovine chondrocytes was -24.26 mV which is less negative than what is reported from most other cells.
4. Other potassium channels are also expressed on the chondrocyte membrane as evidenced by the presence of currents that inactivate over time during the test pulse.
5. Although the effects of NaHS, LNNA, L-arginine and LPS were statistically insignificant, a few trends were noticed. NaHS increased potassium currents and had a small depolarizing effect on the membrane potential. L-arginine decreased the potassium currents and slightly hyperpolarized the membrane. The combined effect of L-arginine and NaHS produced a slight depolarization. LNNA caused a depolarization of the membrane.

6. LPS decreased the currents in majority of cells. The first exposure to LPS caused a hyperpolarization of the cell, whereas later exposures caused a depolarization which was minimally reversed by NaHS.

CONCLUSION

CONCLUSION:

To the best of our knowledge, this study is the first one to demonstrate the effects of the gaseotransmitters – Hydrogen sulphide (H_2S) and Nitric oxide (NO) on the electrophysiological properties of the bovine chondrocyte. The effects of Lipopolysaccharide (LPS) on the electrophysiological properties of the bovine chondrocytes were also studied.

Although the results of the interventions with NaHS, L-arginine, LNNA and LPS were not significant, the trends that have been recorded suggest that both the gaseotransmitters studied (H_2S as well as nitric oxide) may play a significant role in articular cartilage electrophysiology and therefore affect its functioning in health and disease.

Further studies to clarify this role are planned.

LIMITATIONS OF THIS STUDY

LIMITATIONS OF THIS STUDY:

1. Only a small number of bovine chondrocytes were studied in each test condition.
2. A specific inhibitor for H_2S was not used.
3. This study used both fresh and cultured bovine chondrocytes which might have masked the time bound changes.

FUTURE DIRECTION OF THIS STUDY

FUTURE DIRECTION OF THIS STUDY:

The following steps are planned to continue the work on gaseotransmitters

1. The use of a specific H_2S inhibitor in the patch clamp experiments to identify the role of H_2S
2. A higher concentration of H_2S and nitric oxide could be used to obtain clearer results.
3. Different donors of H_2S and nitric oxide can be studied.
4. The effect of gaseotransmitters and LPS on chondrocyte apoptosis is an area that would require more investigation.
5. The differences in the functioning of freshly isolated chondrocytes and cultured chondrocytes needs to be investigated.

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Annexures:

The scans of the following documents are attached.

- Institutional Review Board (IRB) Approval letter.
- IRB fund letter.
- Animal ethics clearance letter.

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

December 18, 2015

Dr. N. Vasanthakumar,
PG Registrar,
Department of Physiology,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research grant project NEW PROPOSAL: (Animal Study)**
The effect of Hydrogen sulphide (H2S) on the electrophysiological properties of bovine articular chondrocytes.
Dr. N. Vasanthakumar (Employment Number: 21190), PG Registrar, Physiology, Dr.Vinay Timothy Oommen, Employment Number: 28645 , Physiology, Dr. Anand Bhaskar (Employment Number: 20438), Physiology

Ref: IRB Min No: 9771 [OTHER] dated 03.12.2015

Dear Dr. N. Vasanthakumar,
The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "The effect of Hydrogen sulphide (H2S) on the electrophysiological properties of bovine articular chondrocytes" on December 03rd 2015.

The Committee reviewed the following documents:

1. IRB Application format
2. Cvs of Drs. Vasanthakumar , Vinay Timothy Oommen, Anand Bhaskar
3. No. of documents 1 – 2

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on December 03rd 2015 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Nihal Thomas,
MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Nihal Thomas	MD, MNAMS, DNB(Endo), FRACP (Endo) FRCP(Edin) FRCP (Glasg)	Professor & Head, Endocrinology. Additional Vice Principal (Research), Deputy Chairperson(Research Chairperson), Member Secretary (Ethics Committee), IRB. CMC, Vellore	Internal, Clinician
Dr. RV. Shaji		Professor, Heamatology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS, MCh	Professor, Neurological Sciences, CMC, Vellore	Internal, Clinician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. B. J. Prashantham	MA(Counseling Psychology), MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Comm IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Mr. Samuel Abraham	MA, PGDBA, PGDPM, M. Phil, BL.	Sr. Legal Officer, CMC, Vellore	Internal, Legal Expert

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Chairperson, Ethics Committee.

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MD, MNAMS, DNB (Endo), FRACP (Endo), FRCP (Edin), FRCP (Glasg)
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Jayaprakash Muliyil	BSc, MBBS, MD, MPH, Dr PH (Epid), DMHC	Retired Professor, CMC, V	External, Scientist &Epidem
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgica Nursing, CMC, Vellore	Internal, Nurse
Mrs. Sheela Durai	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Comn Health, CMC, Vellore	Internal, Clinician

We approve the project to be conducted in its presented form.

This proposal will also need to be submitted to the Institutional Animal Ethics Committee (IAEC) for approval.

The animal requirements and budget will have to be discussed with the Animal House Staff prior to submission of the proposal to the Institutional Animal Experimentation Committee.

Fluid Grant Allocation:

A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr. NIHAL THOMAS
MD.,MNAMS.,DNB(Endo).FRACP(Endo).FRCP(Edin).FRCP(Glasg)
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 9771 [OTHER] dated 03.12.2015

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD
CHRISTIANMEDICALCOLLEGE,
BAGAYAM, VELLORE 632002, TAMIL NADU, INDIA

Ref: FG/9771/12/2015

March 22, 2016

Mr. Robby Pria Sundersingh
The Treasurer
Christian Medical College,
Vellore.

Dear Mr. Robby Pria Sundersingh,

Sub: **Fluid Research grant project NEW PROPOSAL: (Animal Study)**
The effect of Hydrogen sulphide (H2S) on the electrophysiological properties of bovine articular chondrocytes.
Dr. N. Vasanthakumar (Employment Number: 21190), PG Registrar, Physiology, Dr.Vinay Timothy Oommen, Employment Number: 28645 , Physiology, Dr. Anand Bhaskar (Employment Number: 20438), Physiology

Ref: IRB Min. No. 9771 dated 03.12.2015

The Institutional Review Board at its meeting held on December 03rd 2015 vide IRB Min. No. **9771** accepted the project for *A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/Annual report and subsequent submission of it to the IRB.*

Kindly arrange to transfer the sanctioned amount to a separate account to be operated by Dr. Vasanth Kumar(vasanth.dr@gmail.com) and Dr. Vinay Timothy Oommen (vinayoommen@cmcvellore.ac.in)

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MSW., MD., DM
SECRETARY (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

CC: Dr. Vinay Timothy Oommen, Physiology, CMC, Vellore
File

INSTITUTIONAL ANIMAL ETHICS COMMITTEE
CHRISTIAN MEDICAL COLLEGE, VELLORE

Dr. Alfred Job Daniel
Principal and Chairman
email: princi@cmcvellore.ac.in

Dr. Vinay Timothy Oommen
Secretary
email: vinayoommen@cmcvellore.ac.in

25th February 2016

To
Dr. N Vasanthkumar
PG Registrar
Dept. of Physiology
CMC Vellore

Dear Dr. Vasanth Kumar

Re: Animal Ethics approval for Project "The effect of Hydrogen Sulphide on the elelctrophysiological properties of bovine articular chondrocytes", IRB min no 9771

The above proposal need not be taken up by the Institutional Animal Ethics Committee as long as tissue is being utilized from the slaughter house and no animal is being slaughtered for the purpose of this study

With best wishes,
Yours sincerely,

Dr. Alfred Job Daniel,
Principal & Chairperson
Institutional Animal Ethics Committee

Cc:
Dr. Vinay Timothy Oommen
Secretary, IAEC